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Docket No.: 01017/40451B
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Manfred Brockhaus et al.

Application No.: 08/444,790

Confirmation No.: 5612

Filed: May 19, 1995

Art Unit: 1646

For: HUMAN TNF RECEPTOR

Examiner: Zachary Howard

APPEAL BRIEF

MS Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This Appeal Brief is submitted in support of the Notice of Appeal, mailed in this application on August 27, 2007. This Appeal Brief is accompanied by the fee for filing an Appeal Brief under 37 C.F.R. §1.17(b) and a petition and fee for a four-month extension of time under 37 C.F.R. §1.136(a). Accordingly, this Appeal Brief is timely filed and no further fees are believed due. Any additional required fee may be charged, or any overpayment credited, to Deposit Account No. 13-2855.

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I. REAL PARTY IN INTEREST

The real parties in interest for this appeal are Hoffman-LaRoche Inc. (the owner by virtue of an assignment from the inventors recorded at reel 5467, frame 0352) and Immunex Corporation (the licensee), a wholly owned subsidiary of Amgen Inc.

II. RELATED APPEALS AND INTERFERENCES

There are no other related appeals or interferences.

III. STATUS OF CLAIMS

1. Claims canceled: 1-61, 63-101, 104, 108, 109, 112, 115-118, 122 and 138
2. Claims withdrawn from consideration but not canceled: 139
3. Claims pending: 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, 123-137 and 140-144
4. Claims allowed: none
5. Claims rejected: 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, 123-137 and 140-144
6. Claims on appeal: 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, 123-137 and 140-144

IV. STATUS OF AMENDMENTS

The last entered amendment to the claims was Appellants' amendment mailed October 3, 2006 (entered on November 14, 2006). The after-final Amendment filed on August 2, 2007 was not entered, as noted in the Advisory Action mailed October 9, 2007. There are no other outstanding amendments.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The claimed invention relates to a fusion protein that combines fragments of two different proteins: (a) a TNF-binding soluble fragment of the human 75 kD tumor necrosis factor receptor (p75 TNFR) and (b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain (CH1).

Independent claim 62 recites a protein that specifically binds human TNF (page 5, lines 5-9) and that comprises:

(a) a TNF-binding soluble fragment of an insoluble human TNF receptor, wherein the receptor is characterized by (i) specifically binding human TNF (page 7, lines 13-16), (ii) having a molecular weight of about 75 kD (page 7, lines 18-22), and (iii) comprising SEQ ID NO: 10 (page 8, lines 5-6), and

(b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain (page 11, lines 3-10).

Dependent claim 102, which depends from claim 62, recites that the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) (page 8, line 8) and VFCT (SEQ ID NO: 8) (page 7, line 34).

Dependent claim 103, which depends from claim 102, recites that the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10) (page 8, lines 5-6).

Dependent claim 105, which depends from 62, recites that the human immunoglobulin IgG is IgG₁ (page 11, lines 3-10).

Dependent claim 114, which depends from claims 62, 107, 134 and 135, recites a pharmaceutical composition comprising the protein recited in claim 62, 107, 134 or 135, and a pharmaceutically acceptable carrier material (page 12, lines 10-15).

Dependent claim 119, which depends from claim 62, recites that the protein is purified (page 6, line 3-4).

Dependent claim 120, which depends from claim 62, recites that the protein is produced by CHO cells (page 16, line 28).

Dependent claim 121, which depends from claim 62, recites a protein that consists of portion (a) and portion (b) recited in claim 62 (page 5, lines 5-9, page 7, lines 13-16, page 7, lines 18-22, page 8, lines 5-6, page 11, lines 3-10).

Dependent claim 123, which depends from claim 62, recites that the domains of the constant region of the human immunoglobulin heavy chain consist essentially of the

immunoglobulin amino acid sequence encoded by pCD4H γ 1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSM 5314) or by pCD4-H γ 3 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSM 5523) (page 17, lines 25-29).

Dependent claim 124, which depends from claim 105 described above, recites that the domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4H γ 1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSM 5314) (page 17, lines 25-29).

Dependent claim 137, which depends from claim 105 described above, recites a pharmaceutical composition comprising the protein recited in claim 105, and a pharmaceutically acceptable carrier material (page 12, lines 10-15).

Independent claim 106 recites a protein that specifically binds human TNF (page 5, lines 5-9) and that comprises:

(a) a TNF-binding soluble fragment of an insoluble human TNF receptor, wherein the receptor is characterized by (i) specifically binding human TNF (page 7, lines 13-16), (ii) having a molecular weight of about 75 kD (page 7, lines 18-22); and (iii) comprising SEQ ID NO: 10 (page 8, lines 5-6), SEQ ID NO: 12 (page 8, line 8), SEQ ID NO: 8 (page 7, line 34), SEQ ID NO: 9 (page 8, lines 1-2) and SEQ ID NO: 13 (page 8, line 9), and wherein the soluble fragment comprises SEQ ID NO: 12 and SEQ ID NO: 8 (page 7, line 23 – page 8, line 13); and

(b) all of the domains of the constant region of a human IgG₁ heavy chain other than the first domain of the constant region (page 11, lines 3-10).

Dependent claim 125, which depends from claim 106, recites that the protein is purified (page 6, line 3-4).

Dependent claim 126, which depends from claim 106, recites that the protein is produced by CHO cells (page 16, line 28).

Dependent claim 127, which depends from claim 106, recites a protein that consists of portion (a) and portion (b) recited in claim 106 (page 5, lines 5-9, page 7, lines 13-16, page 7, lines 18-22, page 8, lines 5-6, page 8, line 8, page 7, line 34, page 8, lines 1-2, page 8, line 9, page 7, line 23 –page 8, line 13, page 11, lines 3-10).

Dependent claim 128, which depends from claim 106, recites that the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10) (page 8, lines 5-6).

Independent claim 107 recites a recombinant protein that specifically binds human TNF (page 5, lines 5-9) and that is encoded by a polynucleotide which comprises two nucleic acid subsequences (page 11, lines 3-14):

(a) one of said subsequences encoding a TNF-binding soluble fragment of an insoluble human TNF receptor, wherein the receptor is characterized by (i) specifically binding human TNF (page 7, lines 13-16), (ii) having a molecular weight of about 75 kD (page 7, lines 18-22), and (iii) comprising SEQ ID NO: 10 (page 8, lines 5-6); and

(b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain (page 11, lines 3-10).

Dependent claim 110, which depends from claim 107, recites that the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) (page 8, line 8) and VFCT (SEQ ID NO: 8) (page 7, line 34).

Dependent claim 111, which depends from claim 110, recites that the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10) (page 8, lines 5-6).

Dependent claim 113, which depends from claims 107, 110 or 111, recites that the human immunoglobulin heavy chain is IgG₁ (page 11, lines 3-10).

Dependent claim 129, which depends from claim 107, recites that the recombinant protein is purified (page 6, line 3-4).

Dependent claim 130, which depends from claim 107, recites that the protein is produced by CHO cells (page 16, line 28).

Dependent claim 131, which depends from claim 107, recites that the protein consists of (a) the soluble fragment of the receptor (page 7, lines 15-16) and (b) all of the domains of the constant region of the human IgG₁ heavy chain other than the first domain of the constant region. (page 11, lines 3-10).

Dependent claim 132, which depends from claim 107, recites that the domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSM 5314) or by pCD4-Hγ3 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSM 5523) (page 17, lines 25-29).

Dependent claim 133, which depends from claim 113 described above, recites that the domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by the DNA insert of pCD4Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSM 5314) (page 17, lines 25-29).

Independent claim 134 recites a protein that specifically binds human TNF (page 5, lines 5-9), is produced by CHO cells (page 16, line 28), and consists of:

(a) a TNF-binding soluble fragment of an insoluble human TNF receptor (page 7, lines 15-16), wherein the receptor is characterized by (i) specifically binding human TNF (page 7, lines 13-16), (ii) having a molecular weight of about 75 kD (page 7, lines 18-22), and (iii) comprising SEQ ID NO: 10 (page 8, lines 5-6), wherein the soluble fragment comprises SEQ ID NO: 12 and SEQ ID NO: 8 (page 7, line 23 – page 8, line 13); and

(b) all of the domains of the constant region of a human immunoglobulin IgG₁ heavy chain other than the first domain (page 11, lines 3-10).

Dependent claim 135, which depends from claim 134, recites that the soluble fragment comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10) (page 8, lines 5-6).

Dependent claim 136, which depends from claim 134, recites that the protein is purified (page 6, line 3-4).

Independent claim 140 recites a protein that specifically binds human TNF (page 5, lines 5-9) and that comprises:

(a) a TNF-binding soluble fragment of the amino acid sequence encoded by the cDNA insert of the plasmid deposited with the ATCC on October 17, 2006 under accession number PTA 7942 (page 10, line 33-36 as amended); and

(b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain (page 11, lines 3-10).

Dependent claim 141, which depends from claim 140, recites a protein consisting of the soluble fragment (page 7, lines 15-16) and all the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of said constant region (page 11, lines 3-10).

Dependent claim 142, which depends from claim 140, recites that the protein is expressed by a mammalian host cell (page 16, line 28).

Dependent claim 143, which depends from claim 142, recites that the mammalian host cell is a CHO cell (page 16, line 28).

Dependent claim 144, which depends from claim 142, recites a protein consisting of the soluble fragment (page 7, lines 15-16) and all the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of said constant region (page 11, lines 3-10).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. For the purpose of responding to the written description rejection of claims 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, and 123-137 under 35 U.S.C. § 112, first paragraph, claims are grouped as follows:

1. Claims 62, 102, 103, 105, 107, 110, 111, 113, 114, 119, 120, 123, 124, 129, 130, 132, 133, and 137
2. Claims 106, 125, 126, and 128
3. Claims 121, 131, 134, and 136
4. Claim 127
5. Claim 135

B. For the purpose of responding to the obviousness rejection of claims 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, 125-131 and 134-137 under 35 U.S.C. § 103(a), over Dembic *et al.*, *Cytokine* 2:231-237, 1990 (“Dembic”) in view of Capon, U.S. Patent No. 5,116,964 (“Capon”), claims are grouped as follows:

1. Claims 62, 102, 103, 107, 110, 111, 119, 120, 129, and 130
2. Claims 105 and 113
3. Claims 106, 125, 126 and 128
4. Claim 114
5. Claim 121
6. Claim 127
7. Claims 131 and 134-136
8. Claim 137

C. For the purpose of responding to the rejections of claims 140-144 under 35 U.S.C. § 112, first paragraph, claims are grouped as follows:

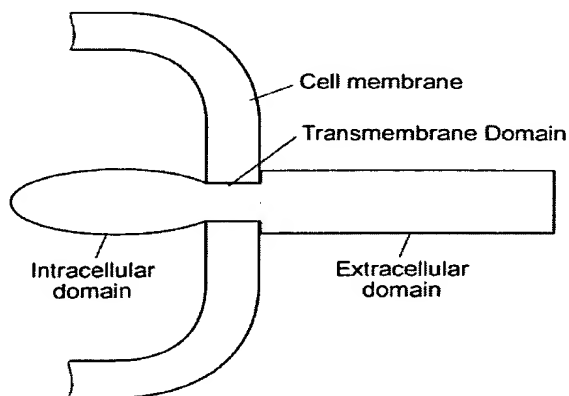
1. The new matter rejection of claims 140-144
2. The enablement rejection of claims 140-144

VII. ARGUMENT

Brief Description of Invention, Background and Specification

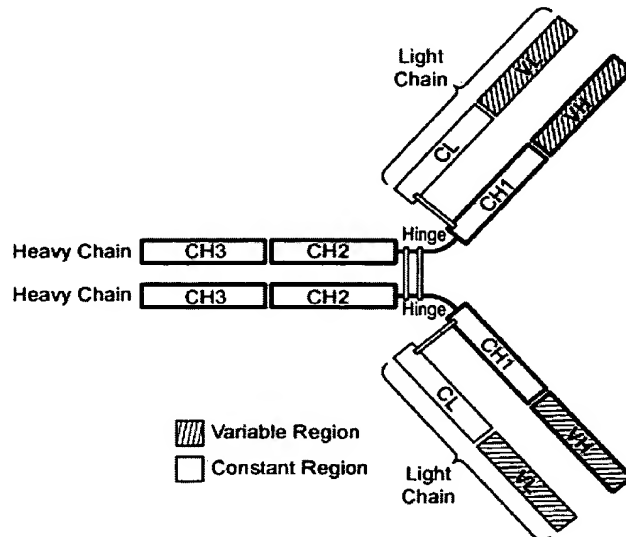
The claimed invention relates to a fusion protein that combines fragments of two different proteins: (a) a TNF-binding soluble fragment of the human 75 kD tumor necrosis factor receptor (p75 TNFR) and (b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain (CH1). As explained immediately below, TNF-binding soluble fragments of p75 TNFR include the extracellular domain and TNF-binding portions thereof. Appellants' invention is not the discovery of p75 TNFR, but rather is the combination of a soluble fragment of p75 TNFR with an IgG heavy chain fragment.

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine which was known in the prior art to mediate a wide range of immunological responses, inflammatory reactions, and anti-tumor effects. TNF had been reported in the art to be a trimer of three identical polypeptides.¹ Two TNF receptors, an approximately 55 kD receptor (p55 TNFR) and an approximately 75 kD/65 kD receptor (p75 TNFR), were known in the art, and the DNA and amino acid sequences for both receptors had been published before the August 31, 1990 priority date of the present application. It was not known at the time whether the TNFRs were monomers, dimers or trimers. Both TNFRs contain an extracellular domain that binds TNF, a transmembrane domain that is embedded in the cell membrane, and an intracellular domain. A protein comprising a TNFR extracellular domain or fragment thereof, without the transmembrane domain, is a soluble, TNF-binding fragment of TNFR. A schematic depiction of a monomeric TNFR is shown below.



¹ Smith & Baglioni, *J. Biol. Chem.*, 262: 6951-6954 (1987) (Smith & Baglioni (1987)) [Appendix B-200].

Complete amino acid sequences for human immunoglobulins, including IgG, were also known in the prior art. Immunoglobulins are naturally occurring antibodies composed of multiple amino acid chains. The IgG type of antibody contains two heavy chains and two light chains. Each IgG heavy chain comprises a variable region (VH) and a constant region composed of the following domains: CH1, hinge, CH2, and CH3 domains. Each light chain comprises a variable region (VL) and a constant region (CL).² Each light chain is linked to a heavy chain, and the two heavy chains are covalently linked to each other by disulfide bonds within the CH1 domain and hinge. A schematic depiction of an IgG immunoglobulin is shown below.



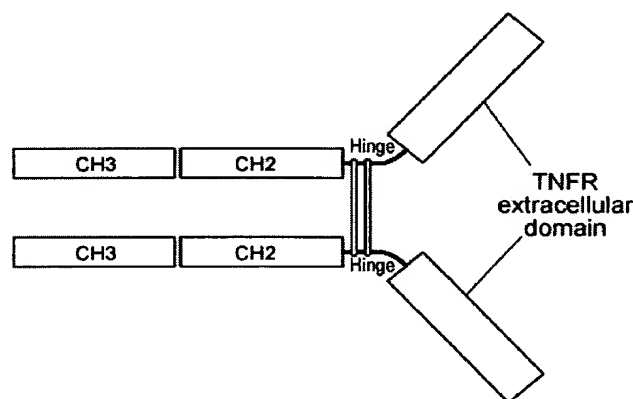
In general, the variable region of an immunoglobulin is responsible for binding antigen, while the constant region is responsible for other functions of immunoglobulins, such as activating antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). Such pro-inflammatory functions are referred to as “effector functions.” ADCC includes the recruiting of white blood cells by antibodies bound to a surface antigen and the destruction of the cells or complexes displaying the antigen.³ CDC is a complex, protein-mediated cascade of events culminating in cell lysis, which can be initiated by antibodies bound to a surface antigen, for example, one displayed

² This general nomenclature is widely used in the art. See, e.g., Capon, U.S. Patent No. 5,116,964 [Appendix B-26].

³ Fundamental Immunology, 2nd Edition, Paul, ed., Raven Press, New York, 1989, at pp. 735-764 (Paul pp. 735-764) [Appendix B-241]

on a foreign cell or virus.⁴ In addition, the constant region of immunoglobulins is known to have a prolonged plasma half life.⁵

The claimed invention relates to a fusion protein comprising a soluble fragment of p75 TNFR (*i.e.*, not membrane-bound) and all of the domains of the constant region of a human IgG heavy chain other than the CH1 domain (retaining the hinge, CH2 and CH3 domains). Due to the natural cysteine disulfide bonding that occurs between heavy chains in the hinge region, the resulting fusion protein is homodimeric. The invention is depicted schematically below.



The present application claims priority to foreign patent applications filed in the German language on September 12, 1989,⁶ March 8, 1990,⁷ April 20, 1990⁸ and August 31, 1990.⁹ Examples 4-6 of the present application describe purification of the two known human TNF receptors, p55 TNFR and p75 TNFR. Example 7 of the present application describes the sequencing of the N-terminus of each purified receptor, as well as sequences of peptides obtained by fragmenting each receptor. Example 8 of the present application describes the cloning of cDNA for p55 TNFR and p75 TNFR, using probes based on the peptide sequences. Examples 9 and 10 of the present application describe recombinant expression of the extracellular domain of the p55 TNFR. Example 11 of the present

⁴ Fundamental Immunology, 2nd Edition, Paul, ed., Raven Press, New York, 1989, at pp. 679-701 (Paul pp. 679-701) [Appendix B-87]

⁵ Capon (1989) [Appendix B-26]

⁶ Swiss Patent Application No. 3319/89 filed September 12, 1989 [Appendix B-273]

⁷ Swiss Patent Application No. 746/90 filed March 8, 1990 [Appendix B-298]

⁸ Swiss Patent Application No. 1347/90 filed April 20, 1990 [Appendix B-344]

⁹ European Patent Application No. 90116707.2 [Appendix B-393], which issued as EP 0417563 (English translation is attached [Appendix B-420])

application describes fusion of this extracellular domain of p55 TNFR to all the domains of the constant region of a human IgG heavy chain other than the CH1 domain.

Figure 1 of the present specification shows full length nucleotide and amino acid sequences of p55 TNFR (SEQ ID NOs: 1 and 2, respectively). Figure 4 of the present specification shows partial nucleotide and amino acid sequences of p75 TNFR (SEQ ID NOs: 3 and 4, respectively) corresponding to amino acids 49-439 of the 439-amino acid mature receptor. The specification also discloses the N-terminal sequence of the first 18 amino acids of p75 TNFR, Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys (SEQ ID NO: 10).¹⁰

Figure 4, which is characterized several times in the specification as a partial sequence,¹¹ first appeared in the April 20, 1990 priority application.¹² The full length human p75 TNFR nucleotide and amino acid sequences subsequently appeared in Smith *et al.* (*Science* 248: 1019-1023, 1990; hereinafter denoted as "Smith (1990)") [Appendix B-211], published in May of 1990. In Smith (1990), Figure 3 and its legend show the signal sequence and the extracellular, transmembrane and intracellular domains of p75 TNFR. Pages 1020-21 of Smith (1990) describe an N-terminal region, amino acids 1-162 of the mature p75 TNFR, that contains the likely TNF-binding site.¹³ Dembic *et al.* (*Cytokine* 2(4), 321-7, 1990; hereinafter denoted as "Dembic" [Appendix B-80]), on which inventors are coauthors, was published in July 1990.¹⁴ Figure 1 of Dembic displays the complete p75 TNFR amino acid sequence and identifies the 235-residue extracellular domain.¹⁵

In the next priority application filing in German on August 31, 1990,¹⁶ Figure 4 was not replaced with a full length sequence. Instead, a citation was added to Smith

¹⁰ Page 8, lines 5-6 of the specification, and page 33, lines 9-19 of the specification in Example 7.

¹¹ See, e.g., page 10, lines 23-26 and page 35, lines 22-23 of the specification.

¹² Swiss Patent Application No. 1347/90 filed April 20, 1990 [Appendix B-344]

¹³ The sentence spanning pages 1020-1021 [Appendix B-80] describes an N-terminal 162 amino acid cysteine-rich region, i.e., amino acids 1-162 of the mature protein. Page 1021, 3rd col. states that "Presumably, it is this NH₂-terminal [cysteine-rich] region that contains the TNF binding site."

¹⁴ Five of the six named inventors on the present application are also co-authors of Dembic. [Appendix B-80]

¹⁵ Figure 1 is at page 232 [Appendix B-80]. The extracellular domain is described at page 233, 1st col.

¹⁶ European Patent Application No. 90116707.2 [Appendix B-393], which issued as EP 0417563

(1990).¹⁷ The citation to Smith (1990) appears at page 10, lines 9-10 of the present specification. That portion, and adjacent portions of the application, are reproduced below:

In addition thereto, the *present invention is also concerned with DNA sequences coding for proteins and soluble or non-soluble fragments thereof, which bind TNF*. Thereunder there are to be understood, for example, DNA sequences coding for non-soluble proteins or soluble as well as non-soluble fragments thereof, which bind TNF, such DNA sequences being selected from the following:

- (a) DNA sequences as given Figure 1 or Figure 4 as well as their complementary strands, or those which include these sequences;
- (b) DNA sequences which hybridize with sequences defined under (a) or fragments thereof;
- (c) DNA sequences which, because of the degeneracy of the genetic code, do not hybridize with sequences as defined under (a) and (b), but which code for polypeptides having exactly the same amino acid sequence.

That is to say, the present invention embraces not only allelic variants, but also those DNA sequences which result from deletions, substitutions and additions from one or more nucleotides of the sequences given in Figure 1 or Figure 4, whereby in the case of the proteins coded thereby there come into consideration, just as before, TNF-BP [TNF binding proteins]. *One sequence which results from such a deletion is described, for example, in [Smith et al.,] Science 248, 1019-1023, (1990).*

There are preferred first of all those DNA sequences which code for such a protein having an apparent molecular weight of about 55 kD, whereby the sequence given in Figure 1 is especially preferred, and sequences which code for non-soluble as well as soluble fragments of such proteins. There are also preferred ***DNA sequences which code for a protein of about 75/65 kD, whereby those which contain the partial cDNA sequences shown in Figure 4 are preferred.*** Especially preferred DNA sequences in this case are the sequences of the open reading frame of nucleotide 2 to 1,177. The peptides IIA, IIC, IIE, IIF, IIG and IIH are coded by the partial cDNA sequence in Figure 4, whereby the insignificant deviations in the experimentally determined amino acid sequences are based on the cDNA-derived sequence with highest probability from the limited resolution of the gas phase sequencing. DNA

¹⁷ Page 6, line 20 of priority application European Pat. Appl. No. 90116707.2.

sequences which code for insoluble as well as *soluble fractions of TNF-binding proteins having an apparent molecular weight of 65 kD/75 kD* are also preferred. DNA sequences for such soluble fragments can be determined on the basis of the amino acid sequences derived from the nucleic acid sequences coding for such non-soluble TNF-BP [TNF binding proteins].

The invention is also concerned with DNA sequences which comprise a combination of two partial DNA sequences, with one of the partial sequences coding for those soluble fragments of non-soluble proteins which bind TNF (see above) and the other partial sequence coding for all domains other than the first domain of the constant region of the heavy chain of human immunoglobulins such as IgG The present invention is also concerned with the recombinant proteins coded by any of DNA sequences described above. [Emphasis added; page 9, line 19 to page 11, line 14]

A. The written description rejection of claims 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, and 123-137 under 35 U.S.C. § 112, first paragraph

The Examiner's rejection of claims 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, and 123-137 for assertedly lacking written description under 35 U.S.C. § 112, first paragraph, should be reversed because (1) Appellants actually possessed and adequately described TNF binding proteins comprising the full length extracellular domain of p75 TNFR, (2) the Examiner's requirement that the specification reiterate sequences known in the prior art is contrary to controlling precedent in factually parallel cases, (3) the Examiner erred by substituting an unsupported personal interpretation of the specification for Appellants' factual declaration evidence regarding what the specification conveyed to the skilled artisan, and (4) the Examiner's overly broad and legally erroneous interpretation of the terms "soluble fragment" of "human" p75 TNFR led to an erroneous conclusion that Appellants lacked adequate written description to support the breadth of the claims.

Brief Statement of Relevant Prosecution History

The claims on appeal were rejected for assertedly lacking written description in the final office action mailed February 23, 2007 ("Final Action"). A major basis for the rejection was the fact that Figure 4 (SEQ ID NO: 4) was missing amino acids 1-48 of the mature human p75 TNFR. Despite acknowledging that Appellants disclosed the correct N-terminal sequence of the purified p75 TNFR, Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-

Pro-Glu-Pro-Gly-Ser-Thr-Cys (SEQ ID NO: 10), the Examiner stated that “[t]ogether, SEQ ID NO: 4 and 10 consist of only a portion of the [p75] TNFR2R extracellular domain (residues 1-7, 9-18 and 49-235).”¹⁸ Citing Chan *et al.*, *Science*, 288: 2351-2354, 2000 (“Chan”) [Appendix B-76] as teaching that amino acids 10-54 (the “PLAD domain”) are required for TNF binding, the Examiner stated that the partial sequence of Figure 4 would not bind TNF because it was missing the PLAD domain.¹⁹ Thus, the Examiner asserted that the specification did not describe TNF-binding soluble fragments of human p75 TNFR.

The Examiner stated that “[w]hile the sequence of the *entire extracellular domain of the 75 kD TNF receptor was publicly available in the references of Smith (1990) and Dembic (1990) at the time of filing of the instant application*, there is no description in the instant specification of these specific full-length sequences, or any description that suggests using these full-length sequences in the claimed fusion proteins.”²⁰ The Examiner’s position was further clarified in the Advisory Action, which states that “the application only points to soluble fragments derived from the partial sequence of Figure 4.”²¹

The Examiner interpreted the specification’s statement that “[o]ne sequence which results from such a deletion is described, for example in [Smith (1990)]” as necessarily referring to “deletions made to the nucleotide sequence of Figure 1 or Figure 4.”²² The Examiner continued, “While Smith [1990] refers to the ‘NH₂-terminal 162 amino acids (positions 39 to 200)’ and teaches that ‘[p]resumably, it is this NH₂-terminal region that contains the TNF binding site’ this is a description of a domain found within a longer protein, not a description of deletion(s) made to a nucleotide sequence . . .”²³

In response to Appellants’ argument that the Court of Appeals for the Federal Circuit, *e.g.* in *Falkner v. Inglis*, 448 F.3d 1357, 1368, 79 U.S.P.Q.2d 1001, 1008 (Fed. Cir. 2006), had held that the specification does not need to recite sequences that are known in the

¹⁸ Page 6 of the Final Action. Because the eighth amino acid is an “X” (indicating an unknown or any amino acid), the Examiner stated that SEQ ID NO: 10 matched amino acids 1-7 and 9-18 of human p75 TNFR.

¹⁹ See pages 6-7 of Final Action.

²⁰ Emphasis added; sentence bridging pages 8 and 9 of the Final Action.

²¹ Page 4, 3rd paragraph of the Advisory Action.

²² Page 9 of the Final Action.

²³ Emphasis omitted; sentence bridging pages 9-10 of the Final Action.

art, the Examiner asserted that *Falkner* did not apply because its fact pattern was “significantly different” from the present case.²⁴

The Examiner also asserted in the Final Action that the specification does not provide a representative number of species of the “vast genus” of proteins claimed, which could comprise “the entire extracellular domain or any fragment thereof as small as one amino acid that retains TNF-binding.”²⁵ No evidence was cited to support the assertion that one amino acid would bind TNF. The Examiner further asserted that the term “human” p75 TNFR includes “artificial receptors with one or more amino acid mutations to the sequence of the insoluble human TNF receptor,”²⁶ despite acknowledging that the specification did not define “human” in this manner.²⁷

In response to the Final Action, Appellants submitted a Declaration Under 37 C.F.R. 1.132 of Stewart Lyman, Ph.D (the “Lyman Declaration”) [Appendix B-143] to provide evidence regarding what the specification reasonably conveyed to the skilled artisan. The Examiner entered the declaration but did not consider it sufficient to overcome the rejection. Although the Lyman Declaration is discussed in detail in the advisory office action mailed October 9, 2007 (“Advisory Action”), the Examiner’s repeated basis for rejecting the factual statements therein was his reliance on the absence of the full-length extracellular domain sequence from the specification.²⁸

1. The written description rejection of claims 62, 102, 103, 105, 107, 110, 111, 113, 114, 119, 120, 123, 124, 129, 130, 132, 133, and 137

a. Appellants actually possessed and adequately described TNF binding proteins comprising the full length extracellular domain of p75 TNFR

The claims are directed to a genus of TNF-binding soluble fragments of human p75 TNFR, fused to all of the domains of an IgG heavy chain constant region other than the CH1 domain. Written description for the immunoglobulin portion of the fusion

²⁴ Page 11 of the Final Action

²⁵ Page 12 of the Final Action

²⁶ Page 13 of Final Action.

²⁷ Page 13 of Final Action.

²⁸ The Examiner used the same basis to deny entry of claims where the TNF binding soluble fragment is the extracellular region of the human TNFR. See Advisory Action 4th paragraph.

protein does not appear to be disputed. The rejection was based on the Examiner's insistence that the application can only describe soluble fragments of the sequence of Figure 4, which is missing amino acids 1-48 of the mature p75 TNFR.

The Examiner erred, however, by disregarding embodiments of the invention that Appellants actually possessed and exemplified, and by failing to consider what the specification reasonably conveys to the skilled artisan. It is well settled that the written description requirement does not require literal support, but merely that applicants reasonably convey to one of skill in the art that they were in possession of the invention at the time of filing. *See, e.g., Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 U.S.P.Q.2d 1111, 1116. (Fed. Cir. 1991).

The specification adequately describes to a skilled artisan TNF-binding soluble fragments of full length human p75 TNFR. This is true for a number of reasons, and is further supported by unrebutted declarant testimony:

(a) Data in Examples 4-7 show that Appellants actually possessed a purified protein containing the full-length extracellular domain of p75 TNFR. Example 7 provides peptide sequences from the purified p75 TNFR that correspond to amino acids 1-18, 43-46, 114-117, 278-284, 324-340 and 410-427 of p75 TNFR.²⁹ The Examiner admitted that, of these peptides, SEQ ID NO: 10 corresponds to amino acids 1-18 of the mature receptor.³⁰ This is proof that Appellants' purified protein must have contained the *full-length* extracellular domain (amino acids 1-235).

(b) The specification contemplates soluble and insoluble fragments of the full length purified p75 TNFR, which are explicitly stated to contain *more than* the partial cDNA sequence shown in Figure 4. Page 10, lines 23-26 describes "DNA sequences which code for a protein of about 75/65 kD, whereby those which contain the partial cDNA sequences shown in Figure 4 are preferred." Thus, it is wholly unreasonable for the Examiner to insist that the

²⁹ Peptides IIA, IIB, IIC, IID, IIF and IIG (see specification page 7, line 34 through page 8, line 9) collectively span amino acids 1-18, 43-46, 114-117, 278-284, 324-340 and 410-427 of the full length p75 TNFR sequence as shown in Figure 1 of Dembic [Appendix B-80].

³⁰ Page 6 of Final Action. Because the eighth amino acid is an "X" (indicating an unknown or any amino acid), the Examiner stated that SEQ ID NO: 10 matched amino acids 1-7 and 9-18 of human p75 TNFR.

description of Appellants' invention must be limited to less than they actually possessed and exemplified.

(c) The specification explicitly states at page 14, lines 32-36 that known sequences can be used as the basis for preparing soluble fragments of the TNFRs ("On the basis of the thus-determined sequences and of the already known sequences for certain receptors. . .") The specification provides a partial cDNA sequence of p75 TNFR (Figure 4). Appellants supplied evidence that "it would be clear one of skill in the art that the protein represented by the Figure 4 sequence was the same protein described in Smith." See the Declaration under 37 CFR §1.132 of Dr. Stewart Lyman, paragraph 16 [Appendix B-143]. Thus, information in the specification would have unambiguously led the skilled artisan to the complete published sequence of p75 TNFR.

(d) Moreover, the specification specifically directs the reader to where such "already known sequences" can be found, by citing to the publication of the full-length sequence in Smith (1990) [Appendix B-211] in a manner that clearly conveys that the sequence of Smith (1990) was part of the invention. The Examiner admitted that Smith (1990) teaches the full-length extracellular sequence of p75 TNFR.³¹ Smith (1990) also identifies a TNF-binding soluble fragment within the extracellular domain that contains the likely TNF binding site.³²

(e) The specification describes soluble and insoluble fragments of TNFRs generally, and states that the p55 and p75 TNFRs purified in Examples 4-6 are preferred embodiments. The examples are stated at page 20, lines 27-30 to be illustrative, not limiting. A fusion protein containing the extracellular domain of the p55 TNFR, fused to all of the domains of an IgG heavy chain constant region other than CH1, is specifically exemplified in Example 11. This illustrative example using p55 TNFR applies equally to the other human TNF receptor, p75 TNFR, as taught by the specification.

(f) The prior art cited by the Examiner to support the obviousness rejection, Dembic [Appendix B-80], shows that the inventors in fact possessed the full-length amino

³¹ Paragraph bridging pages 8 and 9 of Final Action.

³² The sentence spanning pages 1020-1021 describes an N-terminal 162 amino acid cysteine-rich region. Page 1021, 3rd col. states that this region contains the TNF binding site. Smith (1990) [Appendix B-211]

acid sequence of mature human p75 TNFR prior to the instant application's August 31, 1990 priority date. Dembic was co-authored by inventors of the application and was published in July 1990. The Examiner admitted that Dembic teaches the full-length extracellular sequence of p75 TNFR.³³

Thus, the specification adequately describes fusion proteins comprising a TNF-binding soluble fragment of full-length human p75 TNFR fused to all the domains of an IgG heavy chain constant region other than CH1, as claimed, and the rejection under 35 U.S.C. § 112, first paragraph should be reversed.

b. The Examiner erred in disregarding controlling case law in factually parallel cases

The Examiner erred by disregarding controlling case law from the Court of Appeals for the Federal Circuit in factually parallel cases, which each held that the specification need not reproduce DNA sequences that were already known in the art to satisfy the written description requirement. The Examiner's repeated reliance on the absence of the full length p75 TNFR sequence from the specification as the basis for a written description rejection, despite admitting that the full length sequence and extracellular domain were known in the art, is thus legal error. *See, e.g., Capon et al. v. Eshhar et al.*, 418 F.3d 1349, 1357, 76 U.S.P.Q.2d 1078, 1084 (Fed. Cir. 2005); *Falkner v. Inglis*, 448 F.3d 1357, 1368, 79 U.S.P.Q.2d 1001, 1008 (Fed. Cir. 2006); and *Monsanto v. Scruggs*, 459 F.3d 1328, 1336, 79 U.S.P.Q.2d 1813, 1818 (Fed. Cir. 2006) ("neither a specific DNA sequence nor a biological deposit is required to meet a written description requirement if the biological material is known and readily available to the public").

In *Capon v. Eshhar*, the claims at issue related to DNA that encodes chimeric proteins comprising the variable region of an antibody fused to the transmembrane and cytoplasmic portions of a receptor. The claims in *Capon* are highly analogous to the claims of the instant application, which also relate to chimeric proteins. In *Capon*, the Examiner and the Board rejected both parties' claims for lack of written description because no chimeric DNA sequence was recited in the respective specifications. On appeal, both parties argued that their invention was the novel combination of DNA segments known in the art, not the

³³ Paragraph bridging pages 8 and 9 of Final Action.

discovery of the DNA segments themselves, and that re-analysis of known sequences was not required.

The Federal Circuit reversed the Board's decision, holding that the Board erred by requiring the specification to reiterate known prior art sequences:

The Board's rule that the nucleotide sequences of the chimeric genes must be fully presented, although the nucleotide sequences of the component DNA are known, is an inappropriate generalization. When the prior art includes the nucleotide information, precedent does not set a *per se* rule that the information must be determined afresh. *Id.* at 1358.

* * *

The Board erred in holding that the specifications do not meet the written description requirement because they do not reiterate the structure or formula or chemical name for the nucleotide sequences of the claimed chimeric genes. *Id.*

In *Falkner*, Inglis' claims at issue were directed to vaccines containing mutant poxvirus in which essential genes were inactivated, but the Inglis specification did not identify, or even incorporate by reference, poxvirus DNA sequence or essential regions thereof. The Board denied Falkner's motion that Inglis' claims lacked written description because the specification (1) did not identify any essential poxvirus genes, (2) did not have working examples of poxvirus, and (3) for the most part described herpesvirus, not poxvirus. There was undisputed testimony that the DNA sequence of the poxvirus genome and the locations of essential regions were known in the art.

The Federal Circuit affirmed the Board's decision, citing its earlier decision in *Capon*. The Court held that "(1) examples are not necessary to support the adequacy of a written description; (2) the written description standard may be met (as it is here) even where actual reduction to practice of an invention is absent; and (3) there is no *per se* rule that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure." *Falkner*, 448 F.3d at 1366, 79 U.S.P.Q.2d at 1007. The Court further stated, "Indeed, the forced recitation of known sequences in patent disclosures would only add unnecessary bulk to the specification. Accordingly we hold that where, as in this case, accessible literature sources clearly provide, as of the relevant date, genes and their nucleotide sequence (here 'essential genes'), satisfaction of the written

description requirement does not require either the recitation or incorporation by reference of such genes and sequences.” *Falkner*, 448 F.3d at 1368, 79 U.S.P.Q.2d at 1008.

The present facts are directly parallel to both cases. In this case, as in *Capon*, the invention is the novel combination of known sequences. Thus, the Examiner erred, as the Board did in *Capon*, by requiring Appellants’ specification to reproduce the full-length sequence of the p75 TNFR since the sequence was already known in the art. The error is particularly egregious because the specification specifically cited the publication of the full-length sequence in Smith (1990) and clearly conveyed that the sequence of Smith (1990) was part of Appellants’ invention.

In this case, as in *Falkner*, there is evidence, which the Examiner has not disputed, that the claimed sequences were known in the art.³⁴ Thus, as in *Falkner*, reiteration of known sequences through actual examples should not be required to satisfy the written description requirement. In the Final Action, the Examiner took the position that *Falkner* does not apply because its fact pattern was “significantly different.” The Examiner distinguished *Falkner* from the present case by asserting that the disclosure in *Falkner* provided working examples of herpes virus that could be applied to the claimed poxvirus vaccine, while “there is nothing disclosed in the instant specification that is analogous to the herpes virus-based vaccine of Inglis, upon which the skilled artisan could rely as guidance to extend the teachings of the specification from a different receptor to the full-length TNF receptor.”³⁵

The Examiner erred in distinguishing these facts. In *Falkner*, Inglis’ specification was directed generally to vaccine virus vectors and stated that poxvirus was a specific example, although the working examples focused on herpesvirus. Similarly, the present specification concerns TNF receptors generally and states that p75 TNFR is a specific example, although the working examples focused on p55 TNFR. In fact, the present case is even more compelling under the reasoning of *Falkner* since the instant specification (1) identifies the precise p75 TNFR by sequence in Figure 4 and by reference to the Smith (1990) reference containing the complete sequence, (2) had a working example of a p55 TNFR

³⁴Sentence bridging pages 8 and 9 of Final Action.

³⁵Page 11 of Final Action.

which was explicitly not limited to the p55 TNFR, and (3) described TNF receptors and their use in particular for the presently claimed invention. Thus, *Falkner*'s fact pattern is analogous to the present case and the Board should hold here, as the Federal Circuit did in *Falkner*, that there is adequate written description of the p75 TNFR invention.

Factual evidence in the Lyman Declaration, paragraph 17 [Appendix B-143], confirms that the skilled artisan would have understood at the time that "[a]lthough the working examples exemplify a fusion protein comprising the entire extracellular region of the 55 kd TNFR, it is readily apparent that the application's description applies equally to the 75 kd TNFR." Factual evidence in the Lyman Declaration, paragraph 16 [Appendix B-143], also confirms that the partial DNA sequence of p75 TNFR in Figure 4 would have been sufficient for a skilled artisan to determine that the protein represented by the Figure 4 sequence was the same as that disclosed in Smith (1990) [Appendix B-211].

For these reasons, the Examiner erred in disregarding the Federal Circuit's decisions in *Capon* and *Falkner*, and the rejection under 35 U.S.C. § 112, first paragraph should be reversed.

c. The Examiner erred by substituting an unsupported personal interpretation of the specification for the factual evidence in the Lyman Declaration regarding what the specification conveyed to the skilled artisan

Appellants submitted the Lyman Declaration [Appendix B-143] in response to the Examiner's unsupported factual assertion in the Final Action that the reference to Smith (1990) [Appendix B-211] at page 10 of the specification "refers solely to nucleotide sequences with deletions of one or more nucleotides to the sequences given in Figure 1 or Figure 4. . .,"³⁶ *i.e.*, sequences that are fragments of amino acids 49-439 of p75 TNFR. This passage from the specification (starting at page 10, lines 9-10) is quoted in its entirety in Section VII above.

The Examiner erred by substituting an unsupported personal interpretation of the specification for the factual evidence in the Lyman Declaration regarding what the specification conveyed to the skilled artisan. Written description is a question of fact, judged

³⁶ Page 9 of the Final Action.

from the perspective of one of skill in the art as of the filing date, and expert factual evidence regarding the question of written description must be considered. *In re Alton*, 76 F.3d 1168, 1174, 37 U.S.P.Q.2d 1578, 1582 (Fed. Cir. 1996). The Examiner's failure to provide evidence to support a factual assertion in the face of a challenge constitutes clear and reversible error. *See Ex parte Natale*, 11 U.S.P.Q.2d 1222, 1226-27 (Bd. Pat. App. & Interf. 1989); *In re Spormann*, 363 F.2d 444, 447, 150 U.S.P.Q. 449, 452 (C.C.P.A. 1966).

The Lyman Declaration provided a number of reasons why the skilled artisan would have understood from the specification that Appellants contemplated use of the full-length sequence of p75 TNFR known in the art. These reasons included an explanation of why the skilled artisan would have looked to publicly available sequences to complete a partial DNA sequence, and why it was illogical to interpret the reference to the full-length sequence of Smith (1990) as referring solely to fragments of the partial sequence of Figure 4. While the Advisory Action did discuss the Lyman Declaration, the Examiner repeatedly dismissed Dr. Lyman's statements by relying on the absence of the full length p75 TNFR extracellular sequence from the specification.

Paragraphs 6-7 of the Lyman Declaration were not disputed.³⁷ Paragraph 6 states that the application is concerned with two TNF receptors, termed "TNF binding proteins," of about 55 kD and 75 kD, and that the existence of these two receptors was known in the art. Paragraph 7 states that the sequence of p55 TNFR is shown in Figure 1, and the partial sequence of p75 TNFR is shown in Figure 4. The Examiner did not dispute the statements in paragraph 8 and 9 that "soluble fragments" can refer either to the extracellular domain of a TNF receptor or to fragments of this domain, consistent with how the term was used in the art.³⁸

Paragraph 11 of the Lyman Declaration, which was not disputed,³⁹ states that the description of immunoglobulin fusion proteins at page 3, lines 35 to page 4, lines 3 of the specification applies to soluble fragments of *either p55 or p75 TNFR*. Paragraphs 10-12 and 17 further explain that the full-length extracellular domain of either TNFR, as exemplified for p55 TNFR in the working examples, is one example of a soluble fragment. Paragraph 17

³⁷ Page 3, 1st paragraph of the Advisory Action.

³⁸ Page 3, 3rd paragraph of the Advisory Action.

³⁹ Page 3, 6th paragraph of the Advisory Action.

cites the statement at page 20, lines 27-30 of the specification that the examples “illustrate details of the invention” and states that “it would be unreasonable to conclude that this description of soluble fragments of TNF binding proteins applied only to the 55 kd TNFR and not the 75 kd TNFR.” Thus, the specification describes using the entire extracellular region of the p75 TNFR, not just the partial extracellular region described in Figure 4. Despite this evidence, the Examiner continued to assert that “[n]owhere does the specification provide the full-length extracellular sequence of the 75 kD protein, or indicate that this sequence is part of the invention.”⁴⁰

The Lyman Declaration provided factual evidence in paragraphs 18-22 showing why the Examiner’s interpretation of the reference to Smith (1990) in the specification at page 10 was “illogical.” The first and last sentences of the paragraph in question are reproduced below:

In addition thereto, *the present invention* is also concerned with DNA sequences coding for *proteins and soluble or non-soluble fragments thereof, which bind TNF*.

* * *

One sequence which results from such a deletion is described, for example, in [Smith *et al.*,] Science 248, 1019-1023, (1990).⁴¹

Figure 4 displays sequence corresponding to amino acids 49-439 of the 439-amino acid mature human p75 TNFR, and is thus missing the N-terminal 48 amino acids of the receptor sequence. Smith (1990) discloses the entirety of the p75 TNFR sequence, *i.e.* amino acids 1-439 and signal sequence.⁴² Smith also discloses two soluble fragments: the extracellular domain of the receptor (amino acids 1-235) and an N-terminal region (amino acids 1-162) that contains the likely TNF binding site.⁴³

Dr. Lyman notes that the specification states at page 10, lines 23-26 that the Figure 4 sequence is a partial DNA sequence. Dr. Lyman states in paragraph 19 that *there is no soluble fragment sequence disclosed in Smith (1990) which would be a fragment of Figure*

⁴⁰ Page 3, 5th paragraph of the Advisory Action.

⁴¹ Emphasis added; page 9, line 19 through page 10, line 10 of the specification

⁴² Figure 3 of Smith (1990). [Appendix B-211]

⁴³ The sentence spanning pages 1020-1021 describes an N-terminal 162 amino acid cysteine-rich region. Page 1021, 3rd col. states that this region contains the TNF binding site. Smith (1990). [Appendix B-211]

4. In other words, the Smith soluble fragment consisting of amino acids 1-235 cannot be a fragment of a sequence corresponding to amino acids 49-439. Likewise, the Smith soluble fragment consisting of amino acids 1-162 cannot be a fragment of a sequence corresponding to amino acids 49-439. Consequently, Dr. Lyman concludes in paragraph 19 that the reference to Smith (1990) *cannot* refer solely to a sequence that is a deletion of Figure 4.

Dr. Lyman further states that the reference to Smith (1990) must be read in the context of the entire paragraph, as well as the context of the entire application. This paragraph at page 10 of the specification commences with a statement that it is describing soluble and non-soluble fragments of TNF binding proteins *that bind TNF*. Thus, he explains in paragraph 20 that the citation to Smith (1990) was a “reference to *whatever soluble or non-soluble fragments of TNF binding proteins were described in the article*.”⁴⁴

After considering this evidence, the Examiner merely repeated his position that “the reference to Smith only suggests using deletions of the sequence found in Figure 4” and “the specification only contemplates fragments that are deletions of the sequence of Figure 4.”⁴⁵ The Examiner’s statements in the Advisory Action wholly failed to address Dr. Lyman’s testimony and reasoning.

The Lyman Declaration provides further factual evidence in paragraphs 13-16 and 23 that the skilled artisan would look to the already-known sequences of Smith (1990) and Dembic [Appendix B-80] to find the complete p75 TNFR sequence. Paragraph 13 quotes the statement at page 14, lines 32-36 of the specification that soluble fragments of the complete sequence can be prepared “[o]n the basis of the thus-determined sequences *and of the already known sequences for certain receptors*. . .”⁴⁶ Dr. Lyman interpreted the reference to using “known sequences” as using those sequences taught in Smith (1990) and Dembic to determine the full length sequence of p75 TNFR, which was standard practice in the art at the time of filing.⁴⁷ Paragraph 16 states that it would be clear to the skilled artisan that the Smith (1990) sequence is the same p75 TNFR referenced in the present specification

⁴⁴ Emphasis added.

⁴⁵ Page 4, 1st paragraph of the Advisory Action.

⁴⁶ Emphasis added.

⁴⁷ See paragraphs 13-14 and 23. See also paragraph 21, which states that at the filing date it was “common practice to use what is published to aid in assembling a full length sequence.” [Appendix B-143]

and in Figure 4. This statement is supported by evidence showing an alignment of the Smith (1990) sequence with the Figure 4 sequence.⁴⁸ After considering this evidence, the Examiner simply repeated that “Figure 4 describes only a partial sequence of the 75 kD receptor” and “the specification does not describe the sequence of the full-length extracellular domain.”⁴⁹

In the Advisory Action, the Examiner mis-quoted page 14, lines 32-36 of the specification and consequently misinterpreted the reference to “known sequences” to be a reference to Figures 1 and 4.⁵⁰ The properly quoted sentence, in the paragraph immediately above, refers to “already known sequences” as an additional source of sequence information beyond the sequences “thus-determined” in the specification. The Examiner also failed to provide objective evidence, in the form of scientific publications in the art, to contradict Dr. Lyman’s factual statement, as a skilled artisan at the time, that it was “common practice” in 1990 to use published sequence to assemble a full length sequence. More than personal opinion should have been provided to support the Examiner’s contrary position. *See In re Spormann*, 363 F.2d 444, 447, 150 U.S.P.Q. 449, 452 (C.C.P.A. 1966) (“if the Patent Office wishes to rely on [specific knowledge in the prior art], it must produce some reference showing what such knowledge consists of”); *In re Ahlert*, 424 F.2d 1088, 1091, 165 U.S.P.Q. 418, 420-21 (CCPA 1970).

The Examiner’s repeated reliance on the absence of the full-length sequence to support his position is legal error under the Federal Circuit’s holdings in *Capon* and *Falkner* for reasons discussed above in section VII.A.1.b. Moreover, the Examiner erred by disregarding Appellants’ declaratory evidence in favor of his unsupported personal interpretation of the specification. Any statements by the Examiner in rebuttal to the factual statements set forth in the Lyman Declaration should have been supported by objective evidence, not simply the Examiner’s own opinions. *See In re Spormann, supra; In re Ahlert, supra. See also Ex parte Natale*, 11 U.S.P.Q.2d 1222, 1226-27 (Bd. Pat. App. & Interf. 1989).

⁴⁸ Exhibit D of the Lyman Declaration [Appendix B-143].

⁴⁹ Page 3, 8th paragraph of the Advisory Action.

⁵⁰ On page 3, 8th paragraph of the Advisory Action, the examiner provides the following inaccurate quotation, which omits the word “and”: “On the basis of the thus-determined sequences of the already known sequences...”

Appellants' declaration evidence must be given consideration and due weight. For example, in *Scripps Research Institute v. Genentech Inc.* 77 U.S.P.Q. 2d 1809 (Bd. Pat. App. & Interf. 2005), the appellants presented an expert declaration in response to a written description rejection to provide evidence that one of skill in the art would have understood that the description of a deleted transmembrane domain of a tissue factor would include tissue factor proteins from which the entire C-terminal region had been deleted. In this exemplary fact scenario, the Board determined that the expert's reasoning of what one of skill in the art would have understood regarding the transmembrane domain deleted proteins to be credible. *Id.* at 1815. The Board should find the Lyman Declaration in this case to be similarly credible.

The Lyman Declaration provides evidence that one of skill in the art, upon reading the specification in view of the knowledge in the art at the time of filing, would believe that the Appellants were in possession of the claimed invention at the time of filing. The Examiner erred by relying on his unsupported personal interpretation of the specification's disclosure and the absence of already-known sequence from the specification to refute the Lyman Declaration, contrary to controlling precedent. For these reasons, the Board should reverse the rejection under 35 U.S.C. § 112, first paragraph.

d. The Examiner's overly broad and legally erroneous interpretation of claim terms led to an erroneous conclusion that Appellants lacked adequate written description to support the breadth of the claims

The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species, *Regents of University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 1568, 43 U.S.P.Q.2d 1398, 1406 (Fed. Cir. 1997), or by disclosure of relevant, identifying characteristics, *i.e.*, structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, *Enzo Biochem, Inc. v. Gen-Probe Inc.*, 323 F.3d 956, 965, 63 U.S.P.Q. 1609, 1613 (Fed. Cir. 2002).

The claims are directed to a genus of TNF-binding soluble fragments of human p75 TNFR, fused to all of the domains of an IgG heavy chain constant region other

than CH1. Written description for the immunoglobulin portion of the fusion protein does not appear to be disputed. The sole focus of the rejection was the Examiner's position that Appellants do not provide a representative number of species to describe the "vast genus" of claimed TNF-binding fragments of p75 TNFR, based on an overly broad and legally erroneous interpretation of the claims, and that Appellants do not provide structural or functional features of the genus.

i. The erroneous claim interpretation

The Examiner erroneously characterized the claims as being directed to a "vast genus" of "soluble fragments" ranging from the entire extracellular domain to a fragment as small as one amino acid that binds TNF.⁵¹ The Examiner also characterized the claims as encompassing any number of additions, deletions or substitutions, because the Examiner interpreted "human" TNFR as including "artificial receptors" containing one or more mutations.⁵²

The interpretation of TNF-binding "soluble fragments" as including only "one amino acid" is unreasonable and constitutes a completely unsupportable factual assertion. Smith (1990) [Appendix B-211] states that the TNF-binding site is presumably contained within amino acids 1-162 of the mature p75 TNFR. Data in later-published U.S. Patent No. 5,395,760 [Appendix B-219] confirm that this statement in Smith (1990) is correct, and also show that further truncations (e.g., to 1-142) destroy TNF binding activity. This later-published patent is properly cited for the purpose of showing a fact such as a characteristic of a prior art product. *In re Langer*, 503 F.2d 1380, 1391, 183 USPQ 288, 297 (C.C.P.A. 1974); *In re Wilson*, 311 F.2d 266, 268-269, 135 USPQ 442, 444 (C.C.P.A. 1962).

The data in U.S. Patent No. 5,395,760 show that fragments consisting of amino acids 1-235, 1-184, and 1-163 bound TNF, while a shorter fragment consisting of amino acids 1-142 did not.⁵³ Therefore, one would conclude that fragments shorter than 1-

⁵¹ Page 12 of Final Action

⁵² Page 13 of Final Action.

⁵³ U.S. Patent No. 5,395,760 [Appendix B-219] shows data that:

huTNFRΔ235, consisting of amino acids 1-235 of human p75 TNFR, bound TNF (col. 20, lines 38-68).

hu TNFRΔ185, consisting of amino acids 1-185 of human p75 TNFR, bound TNF (col. 21, lines 5-40).

huTNFRΔ163, consisting of amino acids 1-163 of human p75 TNFR, bound TNF (col. 21, lines 45-68).

huTNFRΔ142, consisting of amino acids 1-142 of human p75 TNFR, did not bind TNF (col. 22-, lines 5-49).

142, for example, 5-142 or 10-142, will not bind TNF. Further, the Examiner previously stated that amino acids 10-54 (the “PLAD domain”) are required for TNF binding and that soluble fragments missing this sequence will not bind to TNF.⁵⁴ The Examiner’s statement thus suggests that some portion of the PLAD domain, amino acids 10-54, is required for TNF binding. Consequently, the Examiner’s interpretation of TNF-binding soluble fragments of p75 TNFR as including only a single amino acid of TNFR is unreasonable.

The Examiner’s interpretation of “human” TNFR has no basis in the specification or the art, and is not supported by any evidence. The Examiner merely provides an unsupported personal interpretation of the term “human.” The Examiner admits that there is no definition of “human” in the specification,⁵⁵ yet concludes that the term must include artificial receptors with one or more mutations. While claims during examination are given their broadest reasonable interpretation consistent with the specification, this interpretation must be *reasonable* and must be *consistent with the specification*. See *In re Buszard*, 504 F.3d 1364, 1366, 84 U.S.P.Q.2d 1749, 1751 (Fed. Cir. 2007) (reversal of Board decision in which claims were interpreted unreasonably broadly for purposes of examination); *Merck v. Teva*, 347 F.3d 1367, 1371, 68 U.S.P.Q.2d 1857, 1860 (Fed. Cir. 2003) (“claims must be construed so as to be consistent with the specification”).

Appellants have stated on the record that the term “human” refers to naturally occurring human p75 TNFR, consistent with the use of this term in the art. For example, Smith (1990) refers in Figure 3 to the sequence of the “human TNF receptor cDNA clone” and notes that it was obtained from a “human” cDNA library.⁵⁶ Similarly, the title of Dembic refers to “Two Human TNF Receptors,” and states that the p75 TNFR clone was obtained from “human” genomic DNA,⁵⁷ similar to the statement in Example 8 of the specification that the p75 TNFR clone was obtained from a “human” library. Therefore, the Examiner’s characterization of “human” as including artificial receptors with one or more mutations is not consistent with the use of these terms in the specification or in the art.

⁵⁴ See pages 6-7 of Final Action.

⁵⁵ Page 13 of Final Action.

⁵⁶ See, e.g., p. 1019, 3rd col. Smith (1990). [Appendix B-211]

⁵⁷ Dembic, page 232, 2nd col. [Appendix B-80]

Appellants submitted further factual evidence that human p75 TNFR is uniquely identifiable by its physical characteristics, *i.e.* binding TNF, having a molecular weight of about 75 kd, and comprising the N-terminal peptide sequence set forth in SEQ ID NO: 10. This evidence, provided to the Examiner during prosecution and reproduced here in Appendix B-195, shows that using SEQ ID NO: 10 to search for matching sequences in a comprehensive national database of all publicly available “human” amino acid sequences only identified the human p75 TNFR sequence or naturally occurring allelic variants thereof.⁵⁸ If SEQ ID NO: 10 matched only human p75 TNFR sequence as of June 14, 2006, then certainly SEQ ID NO: 10 would have matched only human p75 TNFR sequence as of Appellants’ effective filing date, at a time when fewer human sequences were known.

Thus, Appellants’ interpretation of the claims, unlike the Examiner’s, is supported by objective evidence of what the skilled artisan would reasonably conclude from the disclosure.

ii. Appellants provided a representative number of species

The specification contemplates a variety of soluble fragments of TNFR that are capable of binding TNF, including those with amino acid deletions relative to the natural sequence.⁵⁹ Soluble fragments include the extracellular domain, as exemplified for p55 TNFR.⁶⁰ The reader is instructed to test fragments using methods such as the TNF-binding assay of Example 1.⁶¹

The specification also cites Smith (1990) at page 10, lines 9-10 not only for the full-length p75 TNFR sequence that it discloses, but also for any TNF-binding “deletion” or fragment that it discloses. Smith (1990) [Appendix B-211] discloses a TNF-binding fragment of p75 TNFR consisting of amino acids 1-235, *i.e.*, the extracellular domain. The

⁵⁸ Appendix B-195 shows the results of a June 14, 2006 search of a Genbank database that includes all publicly available, non-redundant, human Genbank CDS translations (coding amino acid sequence). Over 3.5 million sequences were queried with SEQ ID NO: 10 using the BLASTP search function, and all of the sequences that contained an exact match to SEQ ID NO: 10 are noted to be human p75 TNFR sequence or naturally occurring allelic variants thereof.

⁵⁹ See, *e.g.*, page 6, line 30 to page 7, line 6; page 7, lines 13-22; page 10, lines 1-8; and page 14, lines 32-36 of the specification.

⁶⁰ Examples 9-11 (pages 36-43) of the specification.

⁶¹ Page 7, lines 1-6 of the specification.

Examiner does not dispute the factual statement of Dr. Lyman that the skilled artisan would have expected the full-length extracellular region of TNFR to bind to TNF.⁶² Smith (1990) also discloses a TNF-binding fragment consisting of amino acids 1-162, *i.e.* the N-terminal cysteine-rich region that contains the likely TNF-binding site.⁶³

In addition, Dembic [Appendix B-80] identifies a TNF binding fragment of p75 TNFR consisting of amino acids 1-235, *i.e.*, the extracellular domain.⁶⁴ Dembic further identifies a naturally occurring, soluble fragment of p75 TNFR in human urine that begins at its N-terminus with amino acid 5 of the extracellular domain, *i.e.*, is missing amino acids 1-4.⁶⁵ This fragment in human urine was isolated by its ability to bind TNF and, thus, is a TNF-binding soluble fragment.

When the claims are interpreted properly, it is clear that the specification and the knowledge in the art at the time of filing provided a representative number of species with respect to soluble TNF-binding fragments. For the reasons discussed immediately above in section VII.A.1.d.i, a proper interpretation of the genus of TNF-binding “soluble fragments” of “human” p75 TNFR is that the genus would include the entire extracellular domain (amino acids 1-235), and the TNF-binding domain (amino acids 1-162) disclosed in Smith (1990). As noted above in section VII.A.1.d.i, further truncation at the C-terminus to amino acids 1-142 destroys TNF-binding activity. At the N-terminus, the Examiner’s citation to the Chan article [Appendix B-76] suggests that some portion of the PLAD domain, amino acids 10-54, is required for TNF binding.⁶⁶ TNF-binding soluble fragments of p75 TNFR and the knowledge in the art of TNF-binding species are diagrammatically represented below:

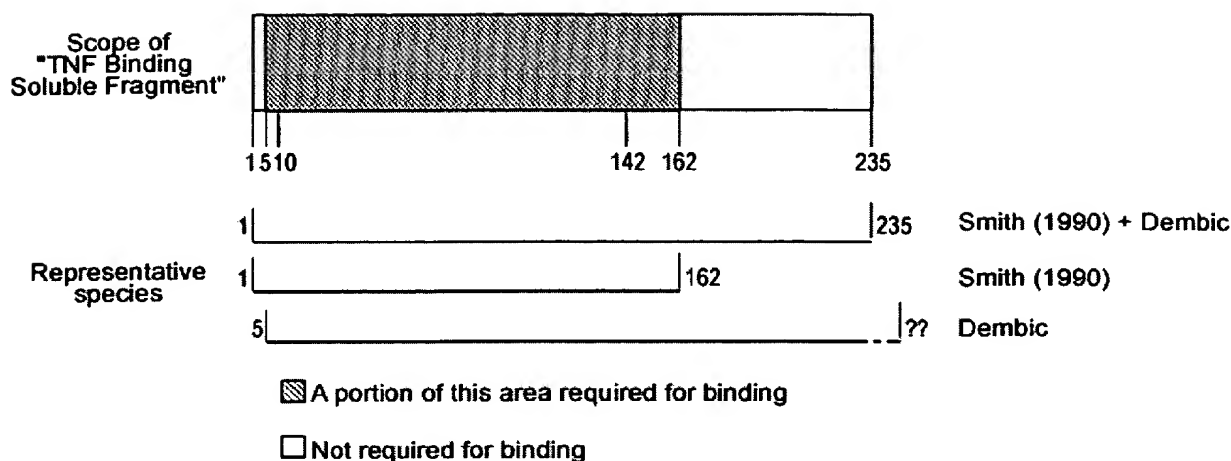
⁶² Page 3, 3rd paragraph of Advisory Action and paragraph 8, page 4 of the Lyman Declaration {Appendix B-143}.

⁶³ Smith (1990), Figure 3 at p. 1021, as well as the text at pp. 1020-1021. 1021, 3rd col. states that the N-terminal cysteine-rich region contains the TNF binding site. [Appendix B-211]

⁶⁴ Dembic at page 233, 1st col. [Appendix B-80]

⁶⁵ Dembic at page 235, 1st col. Dembic states that “We now find that the short NH₂-terminal sequence of the second [TNF] inhibitor matches the V⁵-P⁹ peptide sequence of the 75-kDa TNF receptor (Fig. 1).” Dembic concludes that “these TNF inhibitory peptides therefore are NH₂-terminally truncated, soluble fragments, presumably of the extracellular regions of the two TNF receptors. [Appendix B-80]

⁶⁶ See Chan pg. 2351, right column



Therefore, a representative number of TNF-binding soluble fragment species over the scope of the claimed genus were known as of the filing date.

iii. Appellants provided functional and structural characteristics and a known correlation between function and structure

Alternatively, a genus may be adequately described by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics. *Enzo*, 323 F.3d at 965, 63 U.S.P.Q.2d at 1613. In this case, there is adequate written description because the claims recite the function of TNF binding as well as physical identifying characteristics of human p75 TNFR, and there is a known correlation between structure and function. An assay for TNF binding is provided in Example 1, and use of this assay to test fragments is explicitly described at page 7, lines 1-6 of the specification.

Smith (1990) [Appendix B-211] identifies the extracellular domain of p75 TNFR as amino acids 1-235 of the mature protein. The Examiner does not dispute that the skilled artisan would have expected the full-length extracellular region of TNFR to bind to TNF.⁶⁷ Smith (1990) also identifies the likely TNF binding site as being within amino acids 1-162. Thus, the common structural features of the claimed genus were known in the art at the time of filing, as was the correlation between structure and function. Such a known correlation satisfies the written description requirement. *See Amgen Inc. v. Hoechst Marion*

⁶⁷ Page 3, 3rd paragraph of the Advisory Action.

Roussel, Inc., 314 F.3d 1313, 1332, 65 U.S.P.Q.2d 1385, 1398 (Fed. Cir. 2003) (the written description requirement may be satisfied "if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure").

The claims also recite that the insoluble TNFR is human, has a molecular weight of about 75 kD, and comprises the peptide of SEQ ID NO: 10. These physical and structural characteristics, *i.e.*, size and sequence, provide adequate structural definition for the claimed TNF-binding soluble fragment. As discussed above in section VII.A.1.d.i, Appellants provided factual evidence in Appendix B-195 showing that SEQ ID NO: 10 is sufficient to uniquely identify human p75 TNFR. The Examiner's failure to comment on this evidence in the Final Action was legal error, as the examiner is required to determine patentability based on the totality of the record, which includes all arguments and evidence. *In re Alton*, 76 F.3d 1168, 1176, 37 U.S.P.Q.2d 1578, 1584 (Fed. Cir. 1996).

For all of these reasons, the claim limitations provide the necessary structural and functional definition to provide an adequate written description of the claimed genus, and Appellants have provided a representative number of species that are encompassed by the claimed genus. Therefore, the claimed genus is adequately described and the rejection under 35 U.S.C. § 112, first paragraph should be reversed.

2. The written description rejection of claims 106, 125, 126, and 128

Claims 106, 125, 126 and 128 recite additional peptide sequences within the human p75 TNFR sequence, namely SEQ ID NOS: 8, 9, 12, and 13. While SEQ ID NO: 10 alone for reasons discussed in section VII.A.1.d. would have been sufficient to uniquely identify human p75 TNFR sequence, certainly all of these recited peptide sequences must collectively uniquely identify the human p75 TNFR sequence.

For the reasons discussed in detail above in section VII.A.1, the written description rejection should also be reversed because (1) Appellants actually possessed and adequately described TNF binding proteins comprising the full length extracellular domain of p75 TNFR, (2) the Examiner's requirement that the specification reiterate sequences known in the prior art is contrary to controlling precedent in factually parallel cases, (3) the Examiner erred by substituting an unsupported personal interpretation of the specification for

Appellants' factual declaration evidence regarding what the specification conveyed to the skilled artisan, and (4) the Examiner's overly broad and legally erroneous interpretation of the terms "soluble fragment" of "human" p75 TNFR led to an erroneous conclusion that Appellants lacked adequate written description to support the breadth of the claims.

3. The written description rejection of claims 121, 131, 134 and 136

The scope of claims 121, 131, 134, and 136 differ from that of claim 62 inasmuch as these claims recite "consists of" (claims 121 and 131) or "consisting of" (claim 134). The patentability considerations with respect to these claims are different and must be evaluated separately because these claims are narrower than claim 62. The reduced breadth of the claims further weakens the Examiner's assertion of undue breadth because the genus encompassed by the claim language is correspondingly reduced.

Moreover, the fusion protein of claims 121, 131, 134 and 136 are specifically illustrated by the working example of a fusion protein consisting of a soluble fragment of TNFR fused to a fragment of a human IgG heavy chain constant region lacking the CH1 domain.⁶⁸ While this fusion protein was exemplified with respect to p55 TNFR, the disclosure applies equally to the p75 TNFR for the reasons discussed above in section VII.A.1.d, in the Lyman Declaration and the Federal Circuit's *Falkner* case.

For the reasons discussed in detail above in section VII.A.1, the written description rejection should also be reversed because (1) Appellants actually possessed and adequately described TNF binding proteins comprising the full length extracellular domain of p75 TNFR, (2) the Examiner's requirement that the specification reiterate sequences known in the prior art is contrary to controlling precedent in factually parallel cases, (3) the Examiner erred by substituting an unsupported personal interpretation of the specification for Appellants' factual declaration evidence regarding what the specification conveyed to the skilled artisan, and (4) the Examiner's overly broad and legally erroneous interpretation of the terms "soluble fragment" of "human" p75 TNFR led to an erroneous conclusion that Appellants lacked adequate written description to support the breadth of the claims.

⁶⁸ Example 11, pages 42-43 of the specification.

4. The written description rejection of claim 127

Because claim 127 depends from and incorporates the limitations of claim 106, claim 127 recites additional peptide sequences characterizing the p75 TNFR. However, the scope of claim 127, which recites the language “consists of,” differs from the scope of claim 106. The patentability considerations with respect to claim 127 are thus different and must be evaluated separately because this claim is narrower than claim 106. The reduced breadth of the claim further weakens the Examiner’s assertion of undue breadth because the genus encompassed by the claim language is correspondingly reduced.

Moreover, the fusion protein of claim 127 is specifically illustrated by the working example of a fusion protein consisting of a soluble fragment of TNFR fused to a fragment of a human IgG heavy chain constant region lacking the CH1 domain.⁶⁹ While this fusion protein was exemplified with respect to p55 TNFR, the disclosure applies equally to the p75 TNFR for the reasons discussed above in section VII.A.1.d, in the Lyman Declaration and the Federal Circuit’s *Falkner* case.

Claim 127, like claim 106, also recites additional peptide sequences that are found in the insoluble human p75 TNFR sequence. While SEQ ID NO: 10 alone for reasons discussed in section VIII.A.2.d. would have been sufficient to uniquely identify human p75 TNFR sequence, certainly all of these recited peptide sequences must collectively uniquely identify the human p75 TNF receptor sequence.

For the reasons discussed in detail above in section VII.A.1, the written description rejection should also be reversed because (1) Appellants actually possessed and adequately described TNF binding proteins comprising the full length extracellular domain of p75 TNFR, (2) the Examiner’s requirement that the specification reiterate sequences known in the prior art is contrary to controlling precedent in factually parallel cases, (3) the Examiner erred by substituting an unsupported personal interpretation of the specification for Appellants’ factual declaration evidence regarding what the specification conveyed to the skilled artisan, and (4) the Examiner’s overly broad and legally erroneous interpretation of

⁶⁹ Example 11, pages 42-43 of the specification.

the terms “soluble fragment” of “human” p75 TNFR led to an erroneous conclusion that Appellants lacked adequate written description to support the breadth of the claims.

5. The written description rejection of claim 135

Claim 135 recites the language “consisting of” and also recites additional peptide sequences for the TNF-binding soluble fragment of p75 TNFR, namely SEQ ID NO: 10 (the N-terminal 18 amino acids), SEQ ID NO: 8 and SEQ ID NO: 12. Thus, the patentability considerations with respect to claim 135 differ as well and must be evaluated separately.

Claim 135 is narrower in scope than a claim reciting “comprising.” The reduced breadth of claim 135 further weakens the Examiner’s assertion of undue breadth because the genus encompassed by the claim language is correspondingly reduced.

The fusion protein of claim 135 is specifically illustrated by the working example of a fusion protein consisting of a soluble fragment of TNFR fused to a fragment of a human IgG heavy chain constant region lacking the CH1 domain.⁷⁰ While this fusion protein was exemplified with respect to p55 TNFR, the disclosure applies equally to the p75 TNFR for the reasons discussed above in section VII.A.1.d in the Lyman Declaration and the Federal Circuit’s *Falkner* case.

In addition, claim 135 recites additional peptide sequences that are found in the insoluble human p75 TNFR sequence. While SEQ ID NO: 10 alone for reasons discussed in section VII.A.1.d. would have been sufficient to uniquely identify human p75 TNFR sequence, certainly all of these recited peptide sequences must collectively uniquely identify the human p75 TNF receptor sequence.

For the reasons discussed in detail above in section VII.A.1, the written description rejection should also be reversed because (1) Appellants actually possessed and adequately described TNF binding proteins comprising the full length extracellular domain of p75 TNFR, (2) the Examiner’s requirement that the specification reiterate sequences known in the prior art is contrary to controlling precedent in factually parallel cases, (3) the

⁷⁰ Example 11, pages 42-43 of the specification.

Examiner erred by substituting an unsupported personal interpretation of the specification for Appellants' factual declaration evidence regarding what the specification conveyed to the skilled artisan, and (4) the Examiner's overly broad and legally erroneous interpretation of the terms "soluble fragment" of "human" p75 TNFR led to an erroneous conclusion that Appellants lacked adequate written description to support the breadth of the claims.

B. The obviousness rejection of claims 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, 125-131 and 134-137 under 35 U.S.C. § 103 over Dembic *et al*, Cytokine 2: 231-237, 1990 in view of Capon (US Patent No. 5,116,964)

The Examiner's rejection of claims 62, 102, 103, 105-107, 110, 111, 113, 114, 119-121, 125-131 and 134-137 as assertedly obvious over Dembic and Capon should be reversed because the Examiner has failed to establish a case of obviousness for a number of reasons: (1) the cited art teaches away from combining Dembic with Capon, (2) the Examiner failed to articulate a rationale motivating the selection of the claimed homodimeric fusion protein, in particular, from among the many types of fusion proteins disclosed in Capon, and (3) the Examiner's assertion of a reasonable expectation of success was based on an uncorroborated factual assumption.

Moreover, the rejection should be reversed because the Examiner's refusal to evaluate Appellants' overwhelming evidence of unexpected results is contrary to controlling case law, and Appellants' evidence of unexpected results rebuts any possible case of obviousness.

Brief Statement of Relevant Prosecution History

The rejection of the claims on appeal as assertedly obvious over Dembic [Appendix B-80] and Capon [Appendix B-26] was maintained in the Final Action. The Examiner cited Dembic, the publication co-authored by inventors of the present application, for its disclosure of the full-length amino acid sequence and extracellular domain of the p75 TNFR that forms a TNF-binding soluble fragment. The Examiner acknowledged that Dembic does not teach a fusion of the extracellular domain of the 75 kD TNF receptor with any portion of an immunoglobulin heavy chain constant region.⁷¹ The Examiner cited a secondary reference, Capon [Appendix B-26], as teaching a fusion of a truncated receptor

⁷¹ Page 14 of Final Action.

(not TNFR) to a region of human IgG₁ just upstream of the hinge domain, such that the chimeras contain the hinge, CH2 and CH3 domains of the constant region.⁷²

Capon teaches a vast array of possible hybrid immunoglobulin fusion proteins, including monomeric and homo- or hetero-multimeric forms, among which Capon specifically mentions monomeric, dimeric, trimeric, and tetrameric forms that include various portions of the constant region, such as the entire constant region, hinge-CH2-CH3, or CH2-CH3.⁷³ Capon states that an object of the invention is to provide hybrid immunoglobulin fusion proteins that exhibit increased half-life upon *in vivo* administration.⁷⁴ Capon states that a further object of the invention is to combine the characteristics of a receptor with immunoglobulin effector functions,⁷⁵ which include complement dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). Capon does not disclose any TNFR as a candidate for fusion with an immunoglobulin fragment.

Appellants have argued that Capon teaches away from fusing an anti-inflammatory agent, such as soluble fragments of p75 TNFR, to the pro-inflammatory constant region of an immunoglobulin heavy chain. The Examiner did not dispute that soluble TNF-binding fragments of p75 TNFR are anti-inflammatory and that the constant region of an immunoglobulin heavy chain is pro-inflammatory. Instead, the Examiner stated that the fusion protein was motivated for purposes of affinity purifying TNF, and that, because affinity purification was an *in vitro* use, *in vivo* anti-inflammatory activity was not relevant.⁷⁶ The Examiner further asserted that Capon teaches production of such hybrid immunoglobulins in CHO cells, purification of the resulting fusion protein, and sterile isotonic formulations containing such fusion proteins that would be encompassed by claims to “pharmaceutical compositions.”⁷⁷ The Examiner did not explain why affinity purification compositions, which are not intended for administration to humans, would require sterile isotonic formulations.

⁷²Paragraph bridging pages 14-15 of Final Action.

⁷³ Capon at col. 10, lines 28-30 and col. 13, lines 21-22 and 54. [Appendix B-26]

⁷⁴ Capon at col. 1, lines 8-11, col. 4, lines 38-42, and col. 5, lines 13-20. [Appendix B-26]

⁷⁵ Capon at col. 4, lines 43-47. See also Capon at col. 15, lines 7-8 (C-terminal Fc portion of an antibody contains the effector functions of IgG1). [Appendix B-26]

⁷⁶ Page 15 of Final Action.

⁷⁷ Page 15 of Final Action.

Appellants had submitted a Declaration Under 37 C.F.R. 1.132 of Dr. Werner Lesslauer (“Lesslauer Declaration A”) [Appendix B-129] to rebut the Examiner’s assertion that there was a “reasonable expectation that a dimeric form of the receptor could be used to successfully bind the [TNF] ligand.”⁷⁸ TNF-binding activity would be expected to be relevant to either *in vitro* or *in vivo* uses. The Examiner discussed the declaration in the Final Action but maintained his position regarding expectation of success.

Appellants had also submitted evidence demonstrating that the claimed fusion proteins exhibited unexpected results in a number of different categories relevant to *in vitro* and/or *in vivo* uses of the proteins: (1) the absence or marked reduction in pro-inflammatory effector function of the fusion protein, where the art would have predicted retention of immunoglobulin effector function; (2) lack of ability to form aggregated complexes with TNF; (3) a dramatic 1000-fold increase in TNF neutralization potency, and (4) unexpectedly improved TNF-binding properties, including improved binding affinity and improved kinetic stability.

In the Final Action, the Examiner asserted that the evidence of unexpected results was unpersuasive.⁷⁹ The Examiner’s stated reason for considering the evidence unpersuasive was that it was generated using a fusion protein comprising the full-length extracellular domain of the human p75 TNFR, an embodiment which he believed was not described by the specification.⁸⁰

1. The obviousness rejection of claims 62, 102, 103, 107, 110, 111, 119, 120, 129, and 130

a. The cited art teaches away from combining Capon with Dembic

The primary focus of Capon [Appendix B-26] is providing biologically active molecules for *in vivo* administration. As noted immediately above in the *Brief Statement of Relevant Prosecution History*, Capon states that an object of the invention is to provide hybrid receptor/immunoglobulin fusion proteins that exhibit increased half-life upon *in vivo*

⁷⁸ Page 17 of the Final Action.

⁷⁹ Page 18 of the Final Action.

⁸⁰ Page 18 of the Final Action.

administration,⁸¹ and that retain immunoglobulin effector functions,⁸² such as complement dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC).

The proposed benefit of soluble forms of TNFR was for administration clinically to inhibit TNF, a known pro-inflammatory cytokine. See, e.g., Smith (1990) [Appendix B-211]⁸³. Thus, the disclosure in Capon that the hybrid immunoglobulin fusion proteins were expected to retain the pro-inflammatory effector functions of antibodies teaches away from combining Capon with Dembic. One of ordinary skill in the art would have been discouraged from fusing *an anti-inflammatory* agent, such as soluble fragments of p75 TNFR, to the *pro-inflammatory* constant region of an immunoglobulin heavy chain. "[W]hen the prior art teaches away from combining certain known elements, discovery of a successful means of combining them is more likely to be nonobvious." *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. ___, 127 S. Ct. 1727, 1740, 82 U.S.P.Q.2d 1385, 1395 (2007), citing *United States v. Adams*, 383 U.S. 39, 51-52, 86 S. Ct. 708, 714-715 (1966). As noted below in section VII.B.1.e.i, Appellants' evidence shows that these anti- and pro-inflammatory elements were successfully combined, and exhibited unexpectedly reduced pro-inflammatory effector functions.

The Examiner did not dispute either of these facts, *i.e.*, that soluble fragments of TNFR would be an anti-inflammatory agent, and that the heavy chain constant region is pro-inflammatory. The Examiner instead asserted that a TNFR-immunoglobulin fusion protein was motivated for purposes of affinity purifying TNF. However, the Examiner's proffered articulation of a motivation to combine Dembic and Capon is not rational because affinity purification of TNF was already easily carried out with anti-TNF antibody. See, e.g., Bringman, *Hybridoma*, 6(5):489-507 (1987) (hereinafter "Bringmam") [Appendix B-3], which describes production and use of anti-TNF monoclonal antibodies to purify recombinant TNF-alpha and TNF-beta from bacterial cell lysates. "[R]ejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." *KSR Int'l Co. v. Teleflex, Inc.*, 550 U.S. ___, 127 S. Ct. 1727,

⁸¹ Capon at col. 1, lines 8-11, col. 4, lines 38-42, and col. 5, lines 13-20. [Appendix B-26]

⁸² Capon at col. 4, lines 43-47. See also Capon at col. 15, lines 7-8 (C-terminal Fc portion of an antibody contains the effector functions of IgG1). [Appendix B-26]

⁸³ Smith (1990) at page 1019 and 1022, bottom middle column. [Appendix B-211]

1741, 82 U.S.P.Q.2d 1385, 1396 (2007) (quoting *In re Kahn*, 441 F. 3d 977, 988, 78 U.S.P.Q.2d 1329, 1336 (Fed. Cir. 2006)); *see also Pfizer v. Apotex*, 480 F.3d 1348, 82 U.S.P.Q.2d 1321 (Fed. Cir. 2007).

Thus, a proper case of obviousness has not been articulated by the Examiner because the cited art taught away from combining the anti-inflammatory soluble TNFR with pro-inflammatory immunoglobulin constant region elements. Appellants' discovery of a combination in which the activity of TNFR is enhanced and the pro-inflammatory properties of an immunoglobulin constant region are minimized is therefore nonobvious.⁸⁴

b. There was no motivation to select the claimed fusion proteins which are homodimeric

The Examiner failed to articulate a rationale motivating the selection of the claimed fusion proteins, which are homodimeric, from among the many types of fusion proteins disclosed in Capon.⁸⁵ The selection of a species from a large genus disclosed in a prior art reference is nonobvious. *See In re Baird*, 16 F.3d 380, 382-83, 29 U.S.P.Q.2d 1550, 1552 (Fed. Cir. 1994). As noted above in Section VII.B (*Brief Statement of Relevant Prosecution History*), Capon discloses fusion of receptor fragments to immunoglobulin fragments of varying lengths and with varying conformations, including monomeric, homodimeric, heterodimeric, trimeric, tetrameric, homomultimeric and heteromultimeric forms.⁸⁶ Columns 12-14 of Capon display over one hundred different species [Appendix B-26].

The Examiner's stated motivation for fusing TNFR soluble fragments to immunoglobulin fragments, *i.e.* for the purposes of affinity purifying TNF, does not explain why one of ordinary skill would have selected the claimed *homodimeric* fusion proteins for this purpose. For affinity purification, one of ordinary skill in the art might have disfavored a dimeric form of the fusion protein, because it may have had a spatial geometry that precluded binding to the trimeric TNF ligand,⁸⁷ for the reasons discussed below in section VII.B.1.e.iv.

⁸⁴ See section VII.B.1.e. below.

⁸⁵ See the homodimer schematic at page 13 herein.

⁸⁶ Capon at col. 10, lines 28-30 and col. 13, lines 21-22 and 54. [Appendix B-26]

⁸⁷ Declaration Under 37 C.F.R. 1.132 of Dr. Werner Lesslauer ("Lesslauer Declaration A") [Appendix B-129].

One would have expected a monomeric form lacking disulfide bonding, such as a TNFR-CH₂CH₃ fusion, to bind TNF with greater certainty of success.

Moreover, the Examiner has not articulated a rationale for selecting the claimed fusion proteins from among the many possible types of homodimeric fusion proteins. Example 5 of Capon describes an immunoglobulin fusion protein in which a portion of the hinge region, as well as the CH1 domain, is deleted.⁸⁸ Capon also cites as a preferred embodiment a fusion protein comprising the “entire heavy chain constant region,” including CH1.⁸⁹ In contrast, the claimed constructs contain all of the domains of the constant region of an IgG heavy chain other than CH1.

Thus, a proper case of obviousness has not been articulated because a single species of the many forms of fusion proteins described in Capon is the subject of Appellants’ claims, and no rationale for selecting this particular species has been advanced by the Examiner. Moreover, a protein within the scope of the claims exhibits numerous unexpected results relative to monomeric forms of soluble TNFR. Therefore, the claimed invention represents a nonobvious selection of species.

c. The assertion of reasonable expectation of success was based on an uncorroborated factual assumption

The Examiner asserted a reasonable expectation of success that the claimed dimeric fusion proteins would bind trimeric TNF, based on an uncorroborated factual assumption. This lack of a factual basis to support a finding of reasonable expectation of success, in the face of Appellants’ factual evidence to the contrary, requires reversal of the obviousness rejection.

Appellants had submitted a Declaration Under 37 C.F.R. 1.132 of Dr. Werner Lesslauer (“Lesslauer Declaration A”) [Appendix B-129]. The Lesslauer Declaration A describes that there was uncertainty that the spatial configuration of the *dimeric* TNFR fusion protein would allow it to bind a *trimeric* TNF ligand. The steric distances between the two TNF-binding sites in the dimer and the degree of flexibility required to accommodate the TNF trimer were unknown. Moreover, there was uncertainty that the TNFR portion of the

⁸⁸ Capon at col. 44, lines 63-66. [Appendix B-26]

⁸⁹ Capon at col. 15, lines 9-11 [Appendix B-26]

fusion protein would retain the three-dimensional structure of its TNF-binding site when fused to a relatively large immunoglobulin heavy chain fragment, especially after recombinant production in a host cell. In particular, Lesslauer Declaration A states that “the spatial geometry of the receptor binding site was unknown. Thus, it could have been possible that the fusion with IgG fragments created a spatial structure that would have contained TNF receptor sequences but which, due to its spatial structure, was *completely unable to bind TNF α* . [Emphasis added.]”

To rebut the factual statements in Lesslauer Declaration A, the Examiner cited to Smith & Baglioni (*J. Biol. Chem.* 264:14646-14652, 1989 (“Smith & Baglioni (1989)” [Appendix B-204]) as purported evidence that the TNF trimer was known at the time of filing to bind two receptor molecules. As explained immediately below, this factual assumption is not stated in the cited reference, and, moreover, it is not relevant to the claimed soluble TNFR fusion proteins because all of the data in Smith & Baglioni (1989) is derived from full length TNFRs expressed on a cell surface. From this unsupported assumption, the Examiner improperly inferred a reasonable expectation of success that the claimed dimeric TNFR fusion proteins would bind the trimeric TNF ligand.

Smith & Baglioni (1989) suggest that “TNF receptors on the cell surface consist of high M_r [relative molecular mass] complexes containing *at least two* subunits.”⁹⁰ The data from the reported cross-linking and affinity labeling studies show that TNF binding proteins isolated from solubilized cell membranes form high molecular weight multimeric complexes. The data in Smith & Baglioni (1989) cannot confirm whether the complexes contained a 60 Kd TNF binding protein or a 70 Kd TNF binding protein or both,⁹¹ nor can the data confirm whether the TNFR components within the high molecular weight complexes are dimers of a single subunit or whether these complexes contain additional unidentified proteins.⁹² Hence, no fair reading of Smith & Baglioni (1989) would lead one to conclude that the reference proves that “the TNF ligand trimer was known to bind to two TNF receptors.” Even a decade later, there was still uncertainty regarding the nature of the TNFR complex. For example, Chan [Appendix B-76] reports that “[c]ross-linking the endogenous

⁹⁰ Smith & Baglioni (1989) at page 14650, 2nd column [Appendix B-204]

⁹¹ Smith & Baglioni (1989) at page 14651 2nd column [Appendix B-204]

⁹² Smith & Baglioni (1989) at page 14649, 1st column. [Appendix B-204]

p60 and p80 receptors suggests that *trimers* are a favored conformation.”⁹³ Therefore, the evidence cited by the Examiner does not support his factual assertion that TNF trimers were known to bind two TNF receptors.

Even if it were supported by the cited reference, the examiner’s factual assertion, *i.e.*, that trimeric TNF ligand was known to bind to two TNFR molecules, is not sufficient to support an expectation of success. First, the Examiner does not assert, and Smith & Baglioni (1989) do not show, that any of the TNFR molecules involved in the detected complexes contain any of the sequences recited in the pending claims. Moreover, no evidence was provided that soluble p75 TNFR fused to all of the domains of an IgG heavy chain constant region other than CH1 would be expected to have its TNF-binding portions arranged in a tertiary structure similar to a complex of membrane-bound TNF receptors, which were the only TNF receptors examined in the Smith & Baglioni (1989). The Examiner acknowledged that the structure of the binding site on the TNF receptor was unknown,⁹⁴ a fact which supports Appellants’ declaratory evidence of uncertainty regarding the success in binding.

Since the evidence cited by the Examiner does not support the alleged reasonable expectation of success, it was erroneous for the Examiner to dismiss the declaratory evidence of Dr. Lesslauer as unpersuasive. “Assertions of technical facts in areas of esoteric technology must always be supported by citation to some reference work recognized in the pertinent art.” *In re Alhert* 424 F.2d 1088, 1091, 165 U.S.P.Q. 418, 420-21 (CCPA 1970), *In re Spormann*, 363 F.2d 444, 448, 150 U.S.P.Q. 449, 452 (C.C.P.A.1966). The lack of a factual basis to support a finding of a reasonable expectation of success requires that the obviousness rejection be reversed. *See, e.g., Pfizer v. Apotex*, 480 F.3d 1348, 1361, 82 U.S.P.Q.2d 1321, 1330 (Fed. Cir. 2007) (“[s]ubsumed within the Graham factors is a subsidiary requirement articulated by this court that . . . a skilled artisan would have been motivated to combine the teachings of the prior art references to achieve the claimed invention, and that the skilled artisan would have had a reasonable expectation of success in doing so”).

⁹³ Chan at page 2352, col. 3; emphasis added. [Appendix B-76]

⁹⁴ Page 17 of Final Action.

d. The Examiner legally erred in refusing to evaluate the evidence of unexpected results

Rebuttal evidence with respect to obviousness may include evidence of “secondary considerations” and evidence of unexpected results. *Graham v. John Deere Co.*, 383 U.S. 1, 17, 148 U.S.P.Q. 459, 467 (1966). Appellants’ evidence of unexpected results was generated with fusion proteins that comprised the full length extracellular domain of p75 TNFR, embodiments which the Examiner admitted were encompassed by the language of the claims.⁹⁵ Nevertheless, the Examiner alleged that Appellants’ evidence was insufficient to overcome the obviousness rejection because he believed that fusion proteins comprising the full length extracellular domain were not described by the specification. The Examiner provided no other comments on the merits of the evidence or any indication that he evaluated the evidence at all. The Examiner’s position, thus, appears to be based on the novel legal theory that evidence of unexpected results need not be considered if the Patent Office has taken the position that the relevant claim limitation lacks written description. This was legal error.

The Examiner’s refusal to consider Appellants’ evidence contravenes controlling precedent from the Federal Circuit which holds that the Patent Office cannot disregard evidence presented in rebuttal to an obviousness rejection. *All evidence* of nonobviousness must be considered when assessing patentability. *See In re Soni*, 54 F.3d 746, 750, 34 U.S.P.Q.2d 1684, 1687 (Fed. Circ. 1995); *In Re Sullivan*, 498 F.3d 1345, 1352, 84 U.S.P.Q.2d 1034, 1039 (Fed. Circ 2007). In *Sullivan*, the Board affirmed an obviousness rejection of claims directed to an antibody fragment composition without considering three expert declarations concerning unexpected results. The Board refused to consider the evidence on the ground that it was only relevant to the intended use of the claimed composition. The Federal Circuit held that the Board erred by failing to consider the declarations in a meaningful way. In the instant case, as in *Sullivan*, the Examiner erred by failing to consider Appellants’ evidence of unexpected results.

Thus, it was legal error for the Examiner to refuse to provide meaningful consideration of Appellants’ evidence. Appellants respectfully request that the Board consider the evidence provided and reverse the obviousness rejection.

⁹⁵ Page 10 of Final Action.

e. Unexpected results

Unexpectedly superior properties, unexpectedly different properties, and the absence of expected properties are all relevant factors that can rebut an obviousness rejection. Where the prior art would have predicted the presence of an activity, the absence or significant reduction of that activity is a novel and unexpected property proving nonobviousness. *See Ex parte Mead Johnson & Co.*, 227 U.S.P.Q. 78, 79 (Bd. Pat. App. & Inter. 1985) (absence of a property which a claimed invention would have been expected to possess based on the teachings of the prior art is also evidence of unobviousness).

Similarly, where the prior art would have predicted no improvement in an activity, an improvement in activity constitutes a novel and unexpected property. *In re Corkill*, 711 F.2d 1496, 1499, 226 U.S.P.Q. 1005, 1008 (Fed. Cir. 1985); *In re Chupp*, 816 F.2d 643, 646, 2 U.S.P.Q.2d 1437, 1439 (Fed. Cir. 1987). In some cases, an improvement in activity may be so marked that it may be classified as a different property entirely. *See In re Waymouth*, 499 F.2d 1273, 1276, 182 U.S.P.Q. 290, 293 (CCPA 1974) (marked improvement may be “classified as a difference in kind [of property], rather than one of degree”).

Appellants provided overwhelming evidence of unexpected results in a number of different categories, none of which was considered by the Examiner. Appellants’ evidence demonstrated that the claimed TNFR-fusion proteins exhibit: (1) an absence or marked reduction in pro-inflammatory effector function, where the art would have predicted retention of immunoglobulin effector function; (2) a lack of ability to form aggregated complexes with TNF; (3) a dramatic 1000-fold increase in TNF neutralization potency; and (4) an increased binding affinity and kinetic stability for TNF, where the prior art predicted no change in affinity or kinetic stability.

These unexpected results with respect to TNF binding, TNF neutralization activity, and lack of pro-inflammatory effector function are relevant to *in vitro* and/or *in vivo* uses of the claimed compositions. Even if the unexpected results are only relevant to an intended use, they must be considered. *In re Sullivan, supra* (Board’s refusal to consider evidence relevant to intended use was error). When considered and given due weight, Appellants’ evidence rebuts any possible case of obviousness and requires reversal of the rejection under 35 U.S.C. §103.

i. Unexpectedly absent or drastically reduced pro-inflammatory effector functions

Antibodies are involved in a number of processes that can neutralize foreign, possibly disease-causing, matter. One of the ways in which antibodies neutralize foreign matter, for example, viruses or bacteria, is by recruiting white blood cells to destroy the antibody-coated target cells, a process called antibody-dependent cellular cytotoxicity (ADCC).⁹⁶ ADCC is initiated by binding of the constant region of antibodies to Fc γ R receptors.⁹⁷ Another way in which antibodies mediate destruction of, for example, viruses or bacteria is via a complex, protein-mediated process referred to as complement dependent cytotoxicity (CDC). CDC is initiated by binding of the multimeric complement protein C1q to the constant regions of antibodies.⁹⁸ ADCC and CDC are referred to generally as “effector functions” of antibodies.⁹⁹

Appellants presented evidence generated using a fusion protein within the scope of the claims. Kohno *et al.*, Presentation 1495, poster 271 presented at American College of Rheumatology Annual Meeting, November 13-17, 2005, San Diego, CA (hereafter “Kohno (2005)”) [Appendix B-118]; Khare *et al.*, Poster 715 presented at the Annual Meeting of the Society for Investigative Dermatology (SID), May 3 -5, 2006, Philadelphia, PA (hereafter “Khare (2006)”) [Appendix B-115]; Barone *et al.*, *Arthritis Rheum.*, v42(9) supplement, September 1999 (S90) (“Barone”) [Appendix B-1]. The fusion protein, designated etanercept, consisted of the extracellular domain of p75 TNFR fused to all of the domains of an IgG1 heavy chain constant region other than CH1. Etanercept unexpectedly exhibited drastically reduced, if not completely eliminated, effector function as compared to an anti-TNF antibody. Etanercept bound only weakly to Fc γ R or C1q, proteins that mediate the initiation of ADCC and CDC, respectively. Kohno (2005) [Appendix B-118].¹⁰⁰ Etanercept also exhibited *little or no detectable* ADCC, and *very markedly reduced*

⁹⁶ See page 747 of Paul pp. 735-764 [Appendix B-241]

⁹⁷ See page 738 of Paul pp. 735-764) [Appendix B-241]

⁹⁸ See pp. 681-682 of Paul pp. 679-701 [Appendix-87]

⁹⁹ See Traunecker (*Nature*, 339:68-70, 1989 (“Traunecker”) [Appendix B-216]) and Capon U.S. Patent No. 5,116,964 Col.4, lines 43-47 [Appendix B-26]

¹⁰⁰ Figures 8 and 9 of Kohno (2005) [Appendix B-118] display data from experiments evaluating whether etanercept can bind Fc γ R and C1q in the presence of TNF. A cell line expressing Fc γ RI and Fc γ RII was incubated with radiolabeled etanercept or anti-TNF α antibodies (infliximab and adalimumab) in the presence of the following competitors: an 0.8-fold molar excess of TNF, a 200-fold excess of Fc, or a 200-fold excess of unlabeled etanercept, infliximab, or adalimumab. The results show that etanercept did not bind specifically to

CDC. Khare (2006) [Appendix B-115].¹⁰¹ Another publication reported that etanercept *could not mediate* complement-dependent killing of cells that express TNF. Barone [Appendix B-1].¹⁰²

One of the intended therapeutic uses of the claimed TNFR fusion proteins is to treat inflammatory disorders, by inhibiting or reducing the pro-inflammatory effects of TNF. See page 12, lines 17-21 and page 20, lines 15-18 of the specification. Therefore, the absence of/marked reduction in the pro-inflammatory effector functions of the immunoglobulin portion of the fusion protein is beneficial in the treatment of inflammatory disorders, where promoting inflammation is undesirable.

Appellants also presented evidence showing that, at the time of filing the present application, one of ordinary skill in the art would have expected the claimed immunoglobulin fusion proteins to retain the pro-inflammatory effector activities of the immunoglobulin portion of the fusion. For example, Capon (cited by the Examiner; [Appendix B-26]) teaches that its hybrid immunoglobulin fusion proteins were expected to retain immunoglobulin effector functions,¹⁰³ such as ADCC and CDC. Capon's teaching is confirmed by data reported in pre-filing publications for other immunoglobulin fusions, such as CD4/IgG fusions, which showed that such fusions retained both ADCC and CDC activities. Byrn *et al.* (*Nature*, 344:667-670 (April 1990) ("Byrn") [Appendix B-22]) shows that a fusion protein in which CD4 is fused to the hinge region of IgG retains ADCC activity.¹⁰⁴ Traunecker (*Nature*, 339:68-70, 1989 ("Traunecker") [Appendix B-216]) shows

C1q, whereas each of the antibodies did in the presence of TNF. Figure 9. Similarly, both antibodies showed substantially enhanced binding to cells expressing FcγRI and FcγRII and to C1q in the presence of TNF, whereas etanercept did not. See Figures 8 and 9.

¹⁰¹ Figure 3 of Khare 2006 [Appendix B-115] demonstrates that etanercept mediated *little or no detectable* ADCC compared to infliximab, an anti-TNF antibody which contains an analogous hinge-CH2-CH3 region. Figure 4 shows that etanercept exhibited *markedly less* CDC compared to infliximab. For these assays, MT-3 cells, which express membrane-bound TNF, were incubated with varying concentrations of etanercept or infliximab (an anti-TNFα antibody).

¹⁰² Barone [Appendix B-1] states that "infliximab [an anti-TNF antibody] was able to mediate complement-dependent killing of the TNF-expressing cells (60% lysis at 0.5 mg/mL). In contrast, etanercept was *not able to mediate complement-dependent killing* of the TNF-expressing cells (0% lysis at 1.0 mg/mL)."

¹⁰³ Capon [Appendix B-26] at col. 4, lines 43-47. See also Capon at col. 15, lines 7-8 (C-terminal constant region of an antibody contains the effector functions of IgG1).

¹⁰⁴ Byrn [Appendix B-22] at page 668, first col. states that "CD4 immunoadhesin [a CD4/IgG fusion protein] mediates ADCC towards HIV-infected [cells], but not uninfected, CEM human T-lymphoblastoid cells in a dose-dependent manner (Fig. 2a and b). Soluble recombinant (rCD4) does not mediate ADCC (not shown), but can inhibit cell lysis mediated by CD4 immunoadhesin. . . ."

that a fusion protein in which CD4 is fused to the hinge region of IgG also retains ability to bind Fc γ R and C1q, the proteins that mediate the initiation of ADCC and CDC, respectively.¹⁰⁵ Capon, *Nature*, 337:525-531 (1989) ("Capon (1989)") [Appendix B-69], shows that the CD4-IgG fusion retains the ability to bind well to Fc γ R.¹⁰⁶

Thus, against the expectation in the art as of the application's effective filing date that the claimed TNFR fusion proteins would bind to Fc γ R or C1q and retain pro-inflammatory immunoglobulin effector functions, the observations that a fusion of a soluble fragment of p75 TNFR to the hinge region of IgG1 *lacks* ability to bind Fc γ R or C1q, exhibits *little or no* ADCC, and *is unable to mediate* CDC or exhibits *markedly decreased* CDC, are all unexpected and advantageous properties that render the claimed TNF-binding fusion proteins nonobvious. See *Ex parte Mead Johnson & Co.*, 227 U.S.P.Q. 78, 79 (Bd. Pat. App. & Inter. 1985).

ii. Lack of aggregating ability

Because immunoglobulins are multimeric, multiple immunoglobulin and antigen molecules can form aggregated antibody-antigen complexes. This is consistent with teachings in the art that multivalent binding molecules and multimeric ligands can form aggregated complexes.¹⁰⁷ Aggregation of antibodies greatly enhances effector functions such as ADCC and CDC.¹⁰⁸

¹⁰⁵ Traunecker [Appendix B-216] reported that a CD4/IgG fusion, wherein soluble CD4 was fused to the hinge region of an immunoglobulin heavy chain, retained the ability to bind complement protein C1q and the receptor that initiates ADCC (Fc γ receptor). Traunecker suggests that removal of the CH1 domain does not create major structural alterations in the regions of the CH2 domain responsible for C1q and Fc γ receptor binding. See the data displayed in Figures 3a, 3b and 3c at page 69 of Traunecker.

¹⁰⁶ Capon (1989) [Appendix B-69] at page 528, bottom left col., similarly states that their CD4/Ig fusion bound well to Fc γ R receptor. Although Capon (1989) stated that their CD4/Ig fusion did not bind C1q, the article states that this result was "surprising" (page 529, bottom left col.), indicating that the authors expected to see the results described in Traunecker.

¹⁰⁷ As a general example of a bivalent binding compound, Larsson and Mosbach, *FEBS Lett.* 98(2):333-338 (1979) ("Larsson") [Appendix B-123] shows that a bifunctional NAD compound prepared by covalently linking two NAD compounds (denoted Bis-NAD) was able to complex with and precipitate the tetrameric enzyme lactate dehydrogenase. One technique for detecting the formation of aggregates or complexes is the classic Ouchterlony (double diffusion) test, typically used to detect aggregation of antibodies and antigens. For example, the ligand is placed in the center well of an agarose gel and the ligand binding partner(s) or controls are placed in peripheral wells that are equidistant from the center well. As the ligand and ligand binding partner diffuse towards each other through the gel, they bind and aggregate, causing visible precipitation lines to form in the agarose gel. Results of an Ouchterlony test for bis-NAD and its ligand LDH are shown in Figure 3 at page 337 of Larsson, where precipitation lines can be seen for peripheral wells 1-3 (containing bis-NAD) but not for

Appellants presented evidence showing that two different anti-TNF antibodies, adalimumab and infliximab, form high molecular weight aggregates when combined with TNF, while etanercept, a soluble p75 TNFR fusion protein within the scope of the claims, did not. See Kohno (2005). [Appendix B-118]¹⁰⁹ Etanercept's lack of ability to aggregate is surprising because etanercept, like the anti-TNF antibodies, is divalent. One would have predicted that a divalent TNF binding molecule would form aggregated complexes with trimeric TNF ligand. The lack of ability to aggregate is likely due to the unusual 1:1 binding stoichiometry of the claimed fusion proteins for TNF trimers. Kohno (2005) shows that, when TNF was mixed at varying ratios with etanercept, no complexes were observed in which one molecule of etanercept bound two TNF trimers.¹¹⁰

The absence of an ability to form aggregated complexes with multiple TNF trimers is an unexpected result that is advantageous in treating inflammatory and other disorders. For example, physical proximity of IgG molecules, such as occurs in aggregated complexes, plays an important role in complement activation, which is undesirable in inflammatory disorders.¹¹¹ Further, deposition of antigen:antibody aggregates (also called immune complexes or antigen-antibody complexes) in tissue sites was recognized in the art to be pathogenic. Type III hypersensitivity reactions, serum sickness and autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis were believed to be the result of such immune complexes.¹¹²

wells 4-6 (containing monomeric NAD or buffer only). Thus, the dimeric products form aggregates while the monomeric product does not.

¹⁰⁸ Paul pp. 681-701 at page 681 [Appendix B-87]

¹⁰⁹ See Kohno (2005) [Appendix B-118]. Figure 6 of Kohno 2005 displays results of an Ouchterlony test for three different TNF-binding compounds, etanercept and two anti-TNF antibodies adalimumab and infliximab. In the control experiment (Figure 6A), the center well was filled with a goat anti-Fc antibody which would bind to the common Fc portion of all three TNF-binding compounds. As expected, precipitation lines are formed for all three wells (etanercept, adalimumab and infliximab). In the test experiment (Figure 6B), the center well was filled with TNF. It can be seen that precipitation lines formed for the anti-TNF antibodies (adalimumab and infliximab) but *not* for etanercept, indicating that no TNF-etanercept aggregates were detected.

¹¹⁰ In Kohno (2005) [Appendix B-118], when etanercept and TNF were mixed at varying molar ratios, under the experimental conditions used, size-exclusion chromatography and subsequent determinations of molecular mass and radius by a light scattering detector (SEC-LS) show that etanercept will bind *only one* TNF trimer. See Figures 2 and 5 of Kohno 2005. [Appendix B-118]. When etanercept is present in excess, two molecules of etanercept will bind one TNF trimer (the 300kD complex of Figure 6). However, complexes were not observed in which one molecule of etanercept bound two TNF trimers.

¹¹¹ Fundamental Immunology, 2nd Edition, Paul, ed., Raven Press, New York, 1989, at pp. 679-701. [Appendix B-87]

¹¹² Immunology, 1st Edition, Klein ed., 1990, pp. 446-447 [Appendix B-112]

Thus, against the expectation in the art as of the application's effective filing date that dimeric TNFR fusion proteins would bind two trimeric TNF ligands, the unexpected 1:1 binding stoichiometry and lack of ability to aggregate is an unexpected and advantageous property that renders the claimed TNF-binding fusion proteins nonobvious. *See Ex parte Mead Johnson & Co.* 227 U.S.P.Q. 78, 79 (Bd. Pat. App. & Inter. 1985).

iii. **Unexpected thousand-fold increased potency in TNF neutralization activity**

A soluble p75 TNFR fusion protein within the scope of the claims exhibited an unexpected 50-fold improvement in binding affinity for TNF and a dramatic 1000-fold improvement in TNF neutralizing potency in *in vitro* biological activity assays, compared to the unfused, soluble p75 TNFR (*i.e.*, the monomeric extracellular domain of p75 TNFR alone). Mohler *et al.*, *J. Immunol.*, 151:1548-1561 (1993) (hereafter "Mohler") [Appendix B-181].¹¹³ The 1000-fold increase in TNF neutralizing potency observed *in vitro* for the fusion protein was even higher than would have been predicted on the basis of the 50-fold increased binding affinity, and therefore is of a completely unexpected magnitude that renders the increased potency a different property altogether. *See In re Waymouth*, 499 F.2d 1273, 1276, 182 U.S.P.Q. 290, 293 (CCPA 1974). In contrast, no such increase in potency was observed for other prior art immunoglobulin fragment fusions such as CD4-IgG fusion. Capon (1989) [Appendix B-69] states "Both CD4 immunoadhesins blocked cell killing with the same potency as soluble rCD4."¹¹⁴ Thus, against the expectation in the art, the dramatic increase in potency is an unexpected and advantageous property that renders the claimed TNF-binding fusion proteins nonobvious. *In re Corkill*, 711 F.2d 1496, 1499, 226 U.S.P.Q. 1005, 1008 (Fed. Cir. 1985); *In re Chupp*, 816 F.2d 643, 646, 2 U.S.P.Q.2d 1437, 1439 (Fed. Cir. 1987).

¹¹³ Mohler [Appendix B-181] provides data for a recombinant fusion protein comprising the extracellular domain of the 75 kD TNF receptor (referred to in Mohler as p80) fused to the hinge region of IgG1 (denoted as "sTNFR:Fc" in the article). See page 1554, right column, 2 bottom. The dimeric sTNFR:Fc had about **50 fold higher binding affinity** for TNF than the monomeric soluble TNFR fragment denoted as "sTNFR" (page 1550, col. 2 and Fig. 2A), and the dimeric sTNFR:Fc was about **1000 fold more effective** in neutralizing TNF-induced cytotoxicity in L929 cells (page 1551, left column and Fig. 2B) than the monomeric sTNFR.

¹¹⁴ Capon (1989) [Appendix B-69] in Fig. 5 and at p. 529, bottom right col.

iv. Unexpectedly increased binding affinity and kinetic stability

A different fusion protein within the scope of the claims also exhibited improved binding properties, both in binding affinity and kinetic stability of the bound complexes (how long the complexes remain bound before dissociating). Consistent with the work of Mohler, Lesslauer and coworkers demonstrated that a fusion of soluble p75 TNFR to the hinge region of IgG3 exhibited (a) surprisingly good binding affinity, (b) unexpectedly higher kinetic stability, and (c) improved inhibition of TNF biological activity.¹¹⁵

Such an improvement in binding properties was not observed for prior art immunoglobulin fragment fusions such as CD4-IgG fusion. For example, Capon (1989) demonstrated that a CD4/IgG fusion bound its ligand, gp120, with the same kinetic stability as the soluble, unfused CD4.¹¹⁶ Where the prior art would have predicted no improvement in binding properties for the fusion protein compared to the soluble receptor alone, the observations of increased binding affinity and increased kinetic stability constitute novel and unexpected properties. Particularly in view of Appellants' evidence of uncertainty concerning whether the p75 TNFR fusion proteins would even retain TNF-binding activity at all,¹¹⁷ the observed increased binding affinity and increased kinetic stability are unexpected and advantageous results that render the claimed invention nonobvious. *In re Corkill*, 711 F.2d 1496, 1499, 226 U.S.P.Q. 1005, 1008 (Fed. Cir. 1985); *In re Chupp*, 816 F.2d 643, 646, 2 U.S.P.Q.2d 1437, 1439 (Fed. Cir. 1987).

¹¹⁵ See paragraph 4 of the English translation (Exhibit B; B-137) of Lesslauer Declaration A [Appendix B-129]: "Surprisingly, however, the fusion construct obtained even had an excellent binding activity. In addition, an unexpectedly higher kinetic stability and a surprisingly improved inhibition of the effect of TNF in biological cell culture tests were discovered as well."

Experiment I of Lesslauer Declaration A is a binding study that measured dissociation of the test TNF binding protein from radiolabeled TNF α in the presence of unlabeled TNF α . Dissociation of the dimeric p75sTNFR/IgG fusion was compared to dissociation of the monomeric p75sTNFR. As shown in the figure, at the six-minute time point, essentially all of the TNF α had dissociated from the monomeric p75sTNFR, while only about half of the TNF α had dissociated from the dimeric p75sTNFR/IgG fusion, which indicates that the dimeric product binds for a longer period of time and has a higher kinetic stability than the monomeric product. Experiment II of Lesslauer Declaration A shows that the same dimeric product (at about half the molar concentration of the monomeric product) also produced superior inhibition of TNF biological activity *in vitro*, in an assay measuring TNF-induced proliferation of mononuclear cells.

¹¹⁶ Capon (1989) [Appendix B-69] at p. 526, bottom right col. states "The dissociation constant (Kd) for the interaction of each immunoadhesin [CD4/IgG fusion] with gp120 [the ligand bound by CD4], calculated by Scatchard analysis (Fig. 3a, inset), was indistinguishable from that of soluble rCD4."

¹¹⁷ Lesslauer Declaration A [Appendix B-129] states that "the spatial geometry of the receptor binding site was unknown. Thus, it could have been possible that the fusion with IgG fragments created a spatial structure that would have contained TNF receptor sequences but which, due to its spatial structure, was *completely unable to bind TNF α* [Emphasis added.]"

v. Summary of nonobviousness arguments

As explained above, Appellants respectfully submit that claims 62, 102, 103, 107, 110, 111, 119, 120, 129, and 130 are nonobvious for the following reasons. First, a proper *prima facie* case of obviousness has not been articulated for the following reasons: (1) knowledge in the art taught away from combining Dembic with Capon; (2) no rationale motivating the selection of the claimed homodimeric fusion protein from the many types of fusion proteins disclosed in Capon has been articulated; and (3) the Examiner's assertion of a reasonable expectation of success rests on an uncorroborated factual assumption. Further, even if a proper *prima facie* case had been articulated, Appellants' overwhelming evidence of unexpected results rebuts any possible case of obviousness. Finally, the Examiner's failure to consider this evidence of unexpected results constitutes legal error. Thus, Appellants respectfully request reversal of these rejections for obviousness.

2. The obviousness rejection of claims 105 and 113

Claim 105, dependent from claim 62, recites immunoglobulin IgG₁. Claim 113, dependent from claims 107, 110 or 111, also recites immunoglobulin IgG₁. Appellants' evidence of unexpected results provides an additional and separate basis for patentability with respect to these claims reciting IgG₁. The evidence shows that unexpected results with respect to improved TNF binding and neutralization were observed for different IgG isotypes, both IgG₃ and IgG₁. However, additional evidence of unexpected results was generated for the IgG₁ embodiment, namely the absence of or marked reduction in effector function, and the absence of aggregation ability.

In addition, the rejection of claims 105 and 113 as obvious should be reversed for the following reasons. The Examiner has failed to establish a case of obviousness for a number of reasons: (1) the cited art teaches away from combining Dembic with Capon, (2) the Examiner failed to articulate a rationale motivating the selection of the claimed homodimeric fusion protein, in particular, from among the many types of fusion proteins disclosed in Capon, and (3) the Examiner's assertion of a reasonable expectation of success was based on an uncorroborated factual assumption.¹¹⁸ Moreover, the rejection should be reversed because the Examiner's refusal to evaluate Appellants' overwhelming evidence of

¹¹⁸ See VII.B.1.

unexpected results is contrary to controlling case law, and Appellants' evidence of unexpected results rebuts any possible case of obviousness. Thus, Appellants respectfully request reversal of the rejection of claims 105 and 113 for obviousness.

3. The obviousness rejection of claims 106, 125, 126 and 128

Claims 106, 125, 126 and 128 recite more peptide sequences for the TNF-binding soluble fragment of p75 TNFR than SEQ ID NO: 10, namely SEQ ID NOS: 8, 9, 12, and 13, and also recite immunoglobulin IgG₁. The recitation of further sequence characterizing the TNFR provides for different scope and patentability considerations.

Appellants' evidence of unexpected results provides an additional and separate basis for patentability with respect to these claims reciting IgG₁. The evidence shows that unexpected results with respect to improved TNF binding and neutralization were observed for different IgG isotypes, both IgG₃ and IgG₁. However, additional evidence of unexpected results was generated for the IgG₁ embodiment, namely the absence of or marked reduction in effector function, and the absence of aggregation ability.

In addition, the rejection of claims 106, 125, 126 and 128 as obvious should be reversed for the following reasons. The Examiner has failed to establish a case of obviousness for a number of reasons: (1) the cited art teaches away from combining Dembic with Capon, (2) the Examiner failed to articulate a rationale motivating the selection of the claimed homodimeric fusion protein, in particular, from among the many types of fusion proteins disclosed in Capon, and (3) the Examiner's assertion of a reasonable expectation of success was based on an uncorroborated factual assumption.¹¹⁹ Moreover, the rejection should be reversed because the Examiner's refusal to evaluate Appellants' overwhelming evidence of unexpected results is contrary to controlling case law, and Appellants' evidence of unexpected results rebuts any possible case of obviousness. Thus, Appellants respectfully request reversal of the rejection of claims 106, 125, 126 and 128 for obviousness.

4. The obviousness rejection of claim 114

Claim 114 is additionally nonobvious because the Examiner's rationale for combining Dembic and Capon to show obviousness is completely inapplicable to this claim.

¹¹⁹ See section VII.B.1.

Claim 114 recites pharmaceutical compositions comprising the claimed TNF-binding fusion protein of any of claims 62, 107, 134 or 135 and a pharmaceutically acceptable carrier material. The Examiner's position was that one of ordinary skill in the art was motivated to combine the soluble p75 TNFR fragment of Dembic and with one of the many immunoglobulin fragments of Capon for purposes of affinity purifying TNF.

This rationale of affinity purification completely fails with respect to pharmaceutical compositions. The Examiner asserted that preparation of sterile pharmaceutical compositions was motivated, without explaining why affinity purification compositions need to contain a pharmaceutically acceptable carrier material. The Examiner's assertion is completely unsupported by any evidence, contrary to controlling precedent. *See In re Spormann*, 363 F.2d 444, 447, 150 U.S.P.Q. 449, 452 (C.C.P.A. 1966) ("if the Patent Office wishes to rely on [specific knowledge in the prior art], it must produce some reference showing what such knowledge consists of"); *In re Ahlert*, 424 F.2d 1088, 1091, 165 U.S.P.Q. 418, 420-21 (CCPA 1970).

Moreover, the Examiner's assertion that affinity purification compositions need to contain a sterile pharmaceutically acceptable carrier material is, in Appellants' view, not rational. Thus, the requirement for "articulated reasoning with some rational underpinning to support the legal conclusion of obviousness" is not met for claim 114. *KSR Int'l. Co. v. Teleflex, Inc.*, 550 U.S. ___, 127 S. Ct. 1727, 1741, 82 U.S.P.Q.2d 1385, 1396 (2007) (quoting *In re Kahn*, 441 F. 3d 977, 988, 78 U.S.P.Q.2d 1329, 1336 (Fed. Cir. 2006)); *see also Pfizer v. Apotex*, 480 F.3d 1348, 82 U.S.P.Q.2d 1321 (Fed. Cir. 2007). This constitutes an independent and the separate ground for reversing the obviousness rejection.

In addition, the rejection of claim 114 as obvious should be reversed for the following reasons. The Examiner has failed to establish a case of obviousness for a number of reasons: (1) the cited art teaches away from combining Dembic with Capon, (2) the Examiner failed to articulate a rationale motivating the selection of the claimed homodimeric fusion protein, in particular, from among the many types of fusion proteins disclosed in Capon, and (3) the Examiner's assertion of a reasonable expectation of success was based on

an uncorroborated factual assumption.¹²⁰ Moreover, the rejection should be reversed because the Examiner's refusal to evaluate Appellants' overwhelming evidence of unexpected results is contrary to controlling case law, and Appellants' evidence of unexpected results rebuts any possible case of obviousness. Thus, Appellants respectfully request reversal of the rejection of claim 114 for obviousness.

5. The obviousness rejection of claim 121

The scope of claim 121 differs from that of claim 62, from which it depends, inasmuch as claim 121 recites "consists of." This claim is directed to species in which the TNF-binding soluble fragment of p75 TNFR is fused directly to the domains of the IgG heavy chain constant region, thus excluding embodiments with extra linker sequence(s) between the two segments. Consequently, the scope and patentability considerations with respect to this claim differ. Much of the evidence of unexpected results was generated with a fusion protein that consists of a TNF-binding soluble fragment of p75 TNFR fused to all of the domains of an IgG heavy chain constant region other than CH1.

In addition, the rejection of claim 121 as obvious should be reversed for the following reasons. The Examiner has failed to establish a case of obviousness for a number of reasons: (1) the cited art teaches away from combining Dembic with Capon, (2) the Examiner failed to articulate a rationale motivating the selection of the claimed homodimeric fusion protein, in particular, from among the many types of fusion proteins disclosed in Capon, and (3) the Examiner's assertion of a reasonable expectation of success was based on an uncorroborated factual assumption.¹²¹ Moreover, the rejection should be reversed because the Examiner's refusal to evaluate Appellants' overwhelming evidence of unexpected results is contrary to controlling case law, and Appellants' evidence of unexpected results rebuts any possible case of obviousness. Thus, Appellants respectfully request reversal of the rejection of claim 121 for obviousness.

6. The obviousness rejection of claim 127

The scope of claim 127 differs from that of claim 106, from which it depends, inasmuch as claim 127 recites "consists of." This claim is directed to species in which the

¹²⁰ See section VII.B.1.

¹²¹ See section VII.B.1.

TNF-binding soluble fragment of p75 TNFR is fused directly to the domains of the IgG heavy chain constant region, thus excluding embodiments with extra linker sequence(s) between the two segments. Consequently, the scope and patentability considerations with respect to this claim differ. Much of the evidence of unexpected results was generated with a fusion protein that consists of a TNF-binding soluble fragment of p75 TNFR fused to all of the domains of an IgG heavy chain constant region other than CH1.

In addition, the rejection of claim 127 as obvious should be reversed for the following reasons. The Examiner has failed to establish a case of obviousness for a number of reasons: (1) the cited art teaches away from combining Dembic with Capon, (2) the Examiner failed to articulate a rationale motivating the selection of the claimed homodimeric fusion protein, in particular, from among the many types of fusion proteins disclosed in Capon, and (3) the Examiner's assertion of a reasonable expectation of success was based on an uncorroborated factual assumption.¹²² Moreover, the rejection should be reversed because the Examiner's refusal to evaluate Appellants' overwhelming evidence of unexpected results is contrary to controlling case law, and Appellants' evidence of unexpected results rebuts any possible case of obviousness. Thus, Appellants respectfully request reversal of the rejection of claim 127 for obviousness.

7. The obviousness rejection of claims 131 and 134-136

Claims 131 and 134-136 recite the language "consists of" (claim 131) or "consisting of" (claims 134-136) and also recites IgG₁.

This claim is directed to species in which the TNF-binding soluble fragment of p75 TNFR is fused directly to the domains of the IgG heavy chain constant region, thus excluding embodiments with extra linker sequence(s) between the two segments. Consequently, the scope and patentability considerations with respect to this claim differ. Much of the evidence of unexpected results was generated with a fusion protein that consists of a TNF-binding soluble fragment of p75 TNFR fused to all of the domains of an IgG heavy chain constant region other than CH1.

¹²² See section VII.B.1.

Appellants' evidence of unexpected results provides an additional and separate basis for patentability with respect to these claims reciting IgG₁. The evidence shows that unexpected results with respect to improved TNF binding and neutralization were observed for different IgG isotypes, both IgG₃ and IgG₁. However, additional evidence of unexpected results was generated for the IgG₁ embodiment, namely the absence of or marked reduction in effector function, and the absence of aggregation ability.

In addition, the rejection of claim 131 and 134-136 as obvious should be reversed for the following reasons. The Examiner has failed to establish a case of obviousness for a number of reasons: (1) the cited art teaches away from combining Dembic with Capon, (2) the Examiner failed to articulate a rationale motivating the selection of the claimed homodimeric fusion protein, in particular, from among the many types of fusion proteins disclosed in Capon, and (3) the Examiner's assertion of a reasonable expectation of success was based on an uncorroborated factual assumption.¹²³ Moreover, the rejection should be reversed because the Examiner's refusal to evaluate Appellants' overwhelming evidence of unexpected results is contrary to controlling case law, and Appellants' evidence of unexpected results rebuts any possible case of obviousness. Thus, Appellants respectfully request reversal of the rejection of claims 131 and 134-136 for obviousness.

8. The obviousness rejection of claim 137

Claim 137, which depends from claim 105, is additionally nonobvious because the Examiner's rationale for combining Dembic and Capon to show obviousness is completely inapplicable to this claim. Claim 137 recites pharmaceutical compositions comprising the claimed TNF-binding fusion protein of claim 105 and a pharmaceutically acceptable carrier material. The Examiner's position was that one of ordinary skill in the art was motivated to combine the soluble p75 TNFR fragment of Dembic and with one of the many immunoglobulin fragments of Capon for purposes of affinity purifying TNF.

This rationale of affinity purification completely fails with respect to pharmaceutical compositions. The Examiner asserted that preparation of sterile pharmaceutical compositions was motivated, without explaining why affinity purification

¹²³ See section VII.B.1.

compositions need to contain a pharmaceutically acceptable carrier material. The Examiner's assertion is completely unsupported by any evidence, contrary to controlling precedent. *See In re Spormann*, 363 F.2d 444, 447, 150 U.S.P.Q. 449, 452 (C.C.P.A. 1966) ("if the Patent Office wishes to rely on [specific knowledge in the prior art], it must produce some reference showing what such knowledge consists of"); *In re Ahlert*, 424 F.2d 1088, 1091, 165 U.S.P.Q. 418, 420-21 (CCPA 1970).

Moreover, the Examiner's assertion that affinity purification compositions need to contain a sterile pharmaceutically acceptable carrier material is, in Appellants' view, not rational. Thus, the requirement for "articulated reasoning with some rational underpinning to support the legal conclusion of obviousness" is not met for claim 114. *KSR Int'l. Co. v. Teleflex, Inc.*, 550 U.S. ___, 127 S. Ct. 1727, 1741, 82 U.S.P.Q.2d 1385, 1396 (2007) (quoting *In re Kahn*, 441 F. 3d 977, 988, 78 U.S.P.Q.2d 1329, 1336 (Fed. Cir. 2006)); *see also Pfizer v. Apotex*, 480 F.3d 1348, 82 U.S.P.Q.2d 1321(Fed. Cir. 2007). This constitutes an independent and a separate ground for reversing the obviousness rejection.

In addition, claim 137, like claim 105, is directed to fusions of immunoglobulin IgG1. Appellants' evidence of unexpected results provides an additional and separate basis for patentability with respect to these claims reciting IgG₁. The evidence shows that unexpected results with respect to improved TNF binding and neutralization were observed for different IgG isotypes, both IgG₃ and IgG₁. However, additional evidence of unexpected results was generated for the IgG₁ embodiment, namely the absence of or marked reduction in effector function, and the absence of aggregation ability.

In addition, the rejection of claim 137 as obvious should be reversed for the following reasons. The Examiner has failed to establish a case of obviousness for a number of reasons: (1) the cited art teaches away from combining Dembic with Capon, (2) the Examiner failed to articulate a rationale motivating the selection of the claimed homodimeric fusion protein, in particular, from among the many types of fusion proteins disclosed in Capon, and (3) the Examiner's assertion of a reasonable expectation of success was based on an uncorroborated factual assumption.¹²⁴ Moreover, the rejection should be reversed because the Examiner's refusal to evaluate Appellants' overwhelming evidence of unexpected results

¹²⁴ See section VII.B.1.

is contrary to controlling case law, and Appellants' evidence of unexpected results rebuts any possible case of obviousness. Thus, Appellants respectfully request reversal of the rejection of claim 137 for obviousness.

C. The rejection of claims 140-144 under 35 U.S.C. §112, first paragraph

Brief statement of relevant prosecution history

Claims 140-144 were rejected under 35 U.S.C. §112, first paragraph, for assertedly containing new matter and lacking enablement. Appellants had amended the specification on November 14, 2006 to add reference to a plasmid deposited with the ATCC that contains a DNA sequence encoding insoluble as well as soluble portions of p75 TNFR. At the same time, Appellants added claims 140-144, which recited TNF-binding soluble fragments of the amino acid sequence encoded by the cDNA insert of the deposited plasmid.

Reference to the deposit was inserted at page 10, line 34 of the specification as follows (in which underlining indicates the added text):

DNA sequences which code for insoluble (deposited on October 17, 2006 with the American Type Culture Collection under Accession No. PTA 7942) as well as soluble fractions of TNF-binding proteins having an apparent molecular weight of 65 kD/75 kD are also preferred.

Accompanying the amendment was a Third Declaration of Dr. Werner Lesslauer under 35 U.S.C. §1.132 ("Lesslauer Declaration B") [Appendix B-141]. The declaration stated in paragraph 4 that the deposited material is a DNA construct designated N227 that contains DNA sequence which includes sequence encoding the signal sequence and extracellular domain of human p75 TNFR. The declaration also provided evidence that (1) the deposited material was in Appellants' possession prior to the filing date of the original application, see paragraph 4, and (2) the deposited material is the same as the cDNA identified in the application at page 10, line 34 of the specification, see paragraph 7. Moreover, even without this declaration, it is apparent from the publication by some of the inventors of the entire p75 TNFR sequence (Dembic; [Appendix B-80]) that the inventors were in possession of the entire p75 TNFR sequence prior to the filing date of the original application.

In the Final Action, the Examiner rejected the inserted text as allegedly new matter¹²⁵ and rejected claims 140-144, which reference the deposited material, as assertedly containing new matter.¹²⁶ The Examiner also rejected claims 140-144 as lacking enablement because the specification allegedly does not contain a description of the deposited material sufficient to specifically identify the nature of the deposited sequence.¹²⁷

1. The new matter rejection of claims 140-144 under 35 U.S.C. §112, first paragraph

The rejection of claims 140-144 for asserted new matter should be reversed because Appellants' deposit was in full compliance with applicable case law. The Examiner rejected these claims for assertedly containing new matter, on the grounds that (1) the N227 plasmid was not named in the specification, and (2) N227 cannot be a DNA described in the specification because it contains more sequence (*e.g.*, signal sequence), than is displayed in the partial DNA sequence of Figure 4, which does not contain any signal sequence.¹²⁸

The first reason is not a proper basis for rejecting the claims because neither case law nor the U.S.P.T.O. rules require that the specification include the specific name or designation of the biological preparation in order to deposit the preparation. The 2001 written description guidelines,¹²⁹ cited with approval in *Enzo, supra*, at 964, merely state at footnote 6 that the "description must be sufficient to permit verification that the deposited biological material is in fact that disclosed." In this case, the referenced portion of the specification describes cDNA encoding insoluble and soluble fragments of "TNF binding proteins having an apparent molecular weight of 65 kD/75 kD." Dr. Lesslauer's affidavit evidence is sufficient to permit verification that the deposited N227 plasmid includes DNA encoding insoluble as well as soluble fragments of this 65 kD/75 kD TNF receptor [Appendix B-141].

¹²⁵ Page 22 of the Final Action.

¹²⁶ Page 23 of the Final Action.

¹²⁷ Pages 20-21 of the Final Action. The Final Action also included an enablement rejection because the record was missing a declaration confirming that the deposit was made under the terms of the Budapest Treaty; this aspect of the enablement rejection, however, was withdrawn in the Advisory Action after Appellants submitted such a declaration.

¹²⁸ Pages 23-24 of the Final Action.

¹²⁹ Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, P 1 "Written Description" Requirement, 66 Fed. Reg. 1099 (Jan. 5, 2001) ("Guidelines") at page 1104.

The Federal Circuit's non-precedential decision in *Evans Medical Ltd. v. American Cyanamid Co.*, 215 F.3d 1347, 52 U.S.P.Q.2d (BNA) 1455 (Fed. Cir. 1999) is informative on this point. In *Evans*, the Federal Circuit approved a post-filing deposit of a hybridoma secreting a monoclonal antibody as appropriate and consistent with *In re Lundak* 773 F.2d 1216, 227 U.S.P.Q. 90, 92 (Fed. Cir. 1985) although they noted that the "written description makes no mention of the BB05 monoclonal antibody per se." *Evans*, 52 U.S.P.Q.2d at 1457. U.S. Patent No. 5,237,052 merely refers to the deposit of a hybridoma secreting a "monoclonal immunoglobulin specific for ACAP." The relevant portion at col. 7, lines 30-38 is reproduced below:

Mouse ascitic liquid containing a ***monoclonal immunoglobulin specific for ACAP*** was precipitated at room temperature by the addition of 2 volumes of 27% w/v Na₂SO₄ and left to stand for 2-4 hrs before being sedimented (2000 g for 15 min). The ***hybridoma which secretes the monoclonal immunoglobulin was deposited*** under the Budapest Treaty at the European Collection of Animal Cell Cultures, Porton Down, United Kingdom on Jan. 5, 1990 under accession number 90010501.

Thus, in *Evans*, the description of a monoclonal antibody by its binding specificity for a protein was sufficient to support a deposit of the hybridoma secreting that antibody. By analogy, in the present case, the description of a DNA encoding an identified protein should be sufficient to support a deposit of such a DNA.

The second reason, *i.e.*, that the deposited cDNA cannot have been described in the specification because it contains more sequence than Figure 4 of the application, is also not a proper basis for rejecting the claims because Appellants clearly contemplated a full length p75 TNFR for the reasons noted above in section VII.A.1. Moreover, the presence of a signal sequence in the N227 plasmid cannot form the basis for a new matter rejection where the claims recite the *soluble fragment* portion encoded by the N277 DNA construct and thus the claims exclude the signal sequence. It is understood in the art that the extracellular domain excludes the signal sequence. See, *e.g.*, the identification in Smith (1990) [Appendix B-211] that the extracellular domain commences at amino acid 23, after the 22-amino acid

signal sequence.¹³⁰ The N-terminus of the mature protein is correctly identified in Example 7 of Appellants' specification, and therefore the signal sequence portion of the N227 plasmid is also identifiable.

Thus, the fact that the N227 plasmid contains more DNA sequence than the DNA encoding the extracellular domain is not a proper basis for rejecting the claims for new matter. It is common for deposits related to DNA sequence to include more than the portion of sequence claimed. For example, in *Enzo Biochem, Inc. v. Gen-Probe Inc.*, 323 F.3d 956, 965, 63 U.S.P.Q.2d 1609, 1614 (Fed. Cir. 2002), the biological deposits named in the claims included not only the claimed *N. gonorrhoeae* DNA inserts but also vector sequence from the M13mp8 vector, and bacterial host *E. coli* JM103. See col. 13 lines 27-31 of the patent-at-issue, U.S. Patent No. 4,900,659.¹³¹

Therefore, the rejection of claims 140-144 for asserted new matter should be reversed.

2. The enablement rejection of claims 140-144 under 35 U.S.C. §112, first paragraph

The enablement requirement is that the specification enable one skilled in the pertinent art to make and use the claimed invention. 35 U.S.C. §112, first paragraph. The underlying question to be answered is whether undue or unreasonable experimentation is needed to practice the invention. *In re Wands*, 858 F.2d 731, 737, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988).

The rejection of claims 140-144 for lack of enablement is improper because the Examiner has not established how or why it would require undue experimentation for the skilled artisan to make and/or use the invention of claims 140-144. It is well settled case law that the making of a biological deposit is fully enabling for the material deposited. See *In re Lundak*, 773 F.2d 1216, 1217, 227 U.S.P.Q. 90, 92 (Fed. Cir. 1985); *In re Argoudelis*, 58 C.C.P.A. 769, 771, 434 F.2d 1390, 1390, 168 U.S.P.Q. 99, 100 (C.C.P.A. 1970). One of

¹³⁰ The leader sequence (signal sequence) is underlined in Figure 3 of Smith (1990). See also page 1020. [Appendix B-211]

¹³¹ "These three discrete nucleotide sequences were deposited in the form of a recombinant DNA molecule separately inserted at the Bam HI site of the M13 mp 8 vector, which recombinant molecule has been transformed into the bacterial host *E. coli* JM103." Col. 13, lines 27-31 of U.S. Patent No. 4,900,659.

ordinary skill in the art would have known the location of the extracellular domain from the disclosure of the N-terminal sequence in the present specification, as well as from knowledge in the art such as Smith (1990) [Appendix B-211] and Dembic [Appendix B-80], and would readily have been able to prepare soluble fragments of p75 TNFR from the deposited plasmid.

The Examiner has not presented any evidence showing that it would require undue experimentation to make and use the claimed invention. To the extent that the Examiner's position is based on the absence of the "N227" designation from the specification as originally filed, that is a new matter rejection and not a proper basis for an enablement rejection.

Thus, the rejection of claims 140-144 for assertedly lacking enablement should be reversed.

CONCLUSION

The Examiner's legal and factual errors thus necessitate reversal of all rejections and return of this case to the Examiner for appropriate allowance of the claims.

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APPENDIX A

Claims Involved in the Appeal of Application Serial No. 08/444,790

62. A protein comprising
- (a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human TNF, (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel, and (iii) comprises the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10); and
- (b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region;
- wherein said protein specifically binds human TNF.
102. The protein of claim 62, wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8).
103. The protein of claim 102, wherein the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).
105. The protein of claim 62, wherein said human immunoglobulin IgG heavy chain is IgG₁.
106. A protein comprising
- (a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human

TNF, (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel, and (iii) comprises the amino acid sequences

LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10), LCAP (SEQ ID NO: 12), VFCT (SEQ ID NO: 8), NQPQAPGVEASGAGEA (SEQ ID NO: 9) and VPHLPAD (SEQ ID NO: 13),

wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8); and

(b) all of the domains of the constant region of a human IgG₁ heavy chain other than the first domain of the constant region;

wherein said protein specifically binds human TNF.

107. A recombinant protein encoded by a polynucleotide which comprises two nucleic acid subsequences,

(a) one of said subsequences encoding a human TNF-binding soluble fragment of an insoluble human TNF receptor protein having an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel, said soluble fragment comprising the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10), and

(b) the other of said subsequences encoding all of the domains of the constant region of the heavy chain of a human IgG immunoglobulin other than the first domain of said constant region,

wherein said recombinant protein specifically binds human TNF.

110. The protein of claim 107, wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8).

111. The protein of claim 110, wherein the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

113. The protein of any one of claims 107, 110 or 111, wherein said human immunoglobulin heavy chain is IgG₁.

114. A pharmaceutical composition comprising the recombinant protein of any of claims 62, 107, 134 or 135 and a pharmaceutically acceptable carrier material.

119. The protein of claim 62, wherein the protein is purified.

120. The protein of claim 62, wherein the protein is produced by CHO cells.

121. The protein of claim 62, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of the constant region.

123. The protein of claim 62, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4H γ 1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSM 5314) or by pCD4-H γ 3 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSM 5523).

124. The protein of claim 105, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSM 5314).

125. The protein of claim 106, wherein the protein is purified.

126. The protein of claim 106, wherein the protein is produced by CHO cells.

127. The protein of claim 106, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human IgG₁ heavy chain other than the first domain of the constant region.

128. The protein of claim 106, wherein the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

129. The recombinant protein of claim 107, wherein the recombinant protein is purified.

130. The recombinant protein of claim 107, wherein the recombinant protein is produced by CHO cells.

131. The recombinant protein of claim 107, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human IgG₁ heavy chain other than the first domain of the constant region.

132. The protein of claim 107, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSM 5314) or by pCD4-Hγ3 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSM 5523).

133. The protein of claim 113, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by the DNA insert of pCD4Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSM 5314).

134. A protein consisting of

(a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human TNF, and (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel and (iii) comprises the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10),

wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO:12) and VFCT (SEQ ID NO:8), and

(b) all of the domains of the constant region of a human IgG₁ heavy chain other than the first domain of the constant region,

wherein the protein specifically binds human TNF, and

wherein the protein is produced by CHO cells.

135. The protein of claim 134, wherein the soluble fragment comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

136. The protein of claim 134, wherein the protein is purified.

137. A pharmaceutical composition comprising the recombinant protein of claim 105 and a pharmaceutically acceptable carrier material.

140. A protein comprising

(a) human tumor necrosis factor (TNF) binding soluble fragment of the amino acid sequence encoded by the cDNA insert of the plasmid deposited with the ATCC on October 17, 2006 under accession number PTA 7942,

(b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region;

wherein said protein specifically binds human TNF.

141. The protein of claim 140 consisting of the soluble fragment and all the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of said constant region.

142. The protein of claim 140
wherein the protein is expressed by a mammalian host cell.

143. The protein of claim 142, wherein the mammalian host cell is a CHO cell.

144. The protein of claim 142 consisting of the soluble fragment and all the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of said constant region.

APPENDIX B

Evidence Table of Contents

Page	Description	When filed and when entered or considered
1	“Barone” Barone <i>et al.</i> , <i>Arthritis Rheum.</i> , 42(9) supplement: S90 (September 1999)	Submitted with Amendment and Request for Reconsideration on 10-06- 2006; Considered by Examiner on 02-02-2007
3	“Bringman” Bringman, <i>Hybridoma</i> , 6(5):489-507 (1987)	Submitted with Amendment and Request for Reconsideration on 10-06- 2006; Considered by Examiner on 02-02-2007
22	“Byrn” Byrn <i>et al.</i> , <i>Nature</i> , 344:667-670 (April 1990)	Listed on Form PTO-1449 submitted on 03-07-2000; Considered by Examiner on 07-21-2000
26	“Capon” US Patent No. 5,116,964	Cited by Examiner in Office Action dated 04-03-2006
69	“Capon (1989)” Capon <i>et al.</i> , <i>Nature</i> , 337:525-531 (1989)	Listed on Form PTO-1449 submitted on 05-19-1999. Considered by Examiner on 04-21-00
76	“Chan” Chan <i>et al.</i> , <i>Science</i> , 288: 2351-2354 (2000)	Cited by Examiner in Office Action dated 04-03-2006
80	“Dembic” Dembic <i>et al.</i> , <i>Cytokine</i> , 2(4), 321-7 (1990)	Listed on Form PTO-1449 submitted on 03-07-2000; Considered by Examiner on 07-21-2000
87	“Paul pp. 679-701” <i>Fundamental Immunology</i> , 2nd Edition, Paul, ed., Raven Press, New York, 1989, pp. 769-701	Submitted with Amendment and Request for Reconsideration on 10-06- 2006; Considered by Examiner on 02-02-2007
112	<i>Immunology</i> , 1st Edition, Klein ed., Blackwell Scientific Publications, Cambridge, MA 1990, pp. 446-447	Submitted with Amendment and Request for Reconsideration on 10-06- 2006; Considered by Examiner on 02-02-2007

Page	Description	When filed and when entered or considered
115	“Khare (2006)” Khare <i>et al.</i> Poster 715 presented at the Annual Meeting of the Society for Investigative Dermatology (SID), May 3-5, 2006, Philadelphia, PA	Submitted with Amendment and Request for Reconsideration on 10-06-2006; Considered by Examiner on 02-02-2007
118	“Kohno (2005)” Kohno <i>et al.</i> , Presentation 1495, poster 271 presented at American College of Rheumatology Annual Meeting, November 13-17, 2005	Submitted with Amendment and Request for Reconsideration on 10-06-2006; Considered by Examiner on 02-02-2007
123	“Larsson” Larsson & Mosbach, <i>FEBS Lett.</i> , 98(2):333-338 (1979)	Submitted with Amendment and Request for Reconsideration on 10-06-2006; Considered by Examiner on 02-02-2007
129	“Lesslauer Declaration A” Declaration Under 37 C.F.R. § 1.132 of Dr. Werner Lesslauer	Submitted as Appendix B with Amendment and Request for Reconsideration on 01-18-2005; Entered into the record on 04-05-2005
141	“Lesslauer Declaration B” Third Declaration of Dr. Werner Lesslauer under 35 U.S.C. §1.132	Submitted with Supplemental Amendment in Response to Office Action on 11-14-2006; Entered into the record on 02-23-2007
143	“Lyman Declartion” Declaration Under 37 C.F.R. 1.132 of Stewart Lyman, Ph.D. (with Second Declaration OF Stewart Lyman Ph.D. Under 37 C.F.R. § 1.132)	Submitted with Response to Final Office Action on 08-06-2007; Entered into the record on 10-09-2007
181	“Mohler” Mohler <i>et al.</i> , <i>J. Immunol.</i> , 151:1548-1561 (1993)	Submitted with Amendment and Request for Reconsideration on 01-18-2005; Considered by Examiner on 02-02-2007
195	Results of a search of the Genbank data on June 14, 2206 with SEQ ID NO: 10	Submitted with Response to Nonfinal Office Action on 10-06-2006; Entered into the record on 02-23-2007
200	“Smith & Baglioni (1987)” Smith & Baglioni, <i>J. Biol. Chem.</i> , 262: 6951-6954 (1987)	Submitted with Response to Nonfinal Office Action on 10-06-2006; Considered by Examiner on 02-02-2007
204	“Smith & Baglioni (1989)” Smith & Baglioni, <i>J. Biol. Chem.</i> , 264:14646-14652 (1989)	Cited by Examiner in Final Office Action dated 02-23-2007

Page	Description	When filed and when entered or considered
211	‘Smith (1990)’ Smith <i>et al.</i> , <i>Science</i> , 248:1019-1023 (1990)	Listed on Form PTO-1449 submitted on 09-17-1999; Considered by Examiner on 08-11-2002
216	“Traunecker” Traunecker <i>et al.</i> , <i>Nature</i> , 339:68-70 (1989)	Listed on Form PTO-1449 submitted to PTO on 3-07-2000; Considered by Examiner on 07-21-2000
219	U. S. Patent No. 5,395,760	Cited by Examiner in Office Action dated 02-01-1996
241	“Paul pp. 735-764” Fundamental Immunology, 2 nd Edition, Paul, ed., Raven Press, New York, 1989 pp. 735-764	Submitted with Amendment and Request for Reconsideration on 10-06-2006; Considered by Examiner on 02-02-2007
273	Swiss Patent Application No. 3319/89 filed September 12, 1989	Listed on Transmittal of New Patent Application submitted to PTO on 05-19-1995. Certified Copy was provided in U.S. Application No. 07/580.013
298	Swiss Patent Application No. 746/90 filed March 8, 1990	Listed on Transmittal of New Patent Application submitted to PTO on 05-19-1995. Certified Copy was provided in U.S. Application No. 07/580.013
344	Swiss Patent Application No. 1347/90 filed April 20, 1990	Listed on Transmittal of New Patent Application submitted to PTO on 05-19-1995. Certified Copy was provided in U.S. Application No. 07/580.013
393	European Patent Application No. 90116707.2 filed August 31, 1990, which issued as EP 0417563	Certified Copy was submitted to PTO on 08-06-2007
420	English translation of European Patent Application No. 90116707.2	Appendix B of Second Declaration of Stuart Lyman. Ph.D; Submitted with Response to Final Office Action on 08-06-2007; Entered into the record on 10-09-2007

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Arthritis and Rheumatism

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COMPARATIVE ANALYSIS OF THE ABILITY OF ETANERCEPT AND INFlixIMAB TO LYSE TNF-EXPRESSING CELLS IN A COMPLEMENT DEPENDENT FASHION.

[Abstract Supplement; 1999 Annual Scientific Meeting:
November 13 - 17, 1999; Boston, Massachusetts:
ACR/ARHP Scientific Abstracts: Poster Sessions]

Barone, Dauphine; Krantz, Carol; Lambert, Dina; Maggiora, Kathy; Mohler, Kendall

Seattle, WA

Abstract 116

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Recent History

COMPARATIVE ANALYSIS OF T...

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Published reports 2 have demonstrated that infliximab (REMICADE®), a mouse-human chimeric antibody to TNF, is able to kill some TNF-expressing cells *in vitro* in the presence of complement (C'). Those experiments utilized cells that were unable to shed TNF and thus expressed high levels of cell-surface TNF. We have examined the ability of etanercept (ENBREL®), a soluble fusion protein consisting of two human p75 TNF receptors linked to the Fc region of human IgG1, to kill TNF-expressing cells in the presence of C'. To directly assess the ability of etanercept to kill TNF-expressing cells, cDNAs containing the sequence for a mutated human TNF gene were obtained. The mutated genes encoded a sequence that greatly reduced the amount of TNF shed from the cell surface. These genes were transfected into CHO cells, and cell lines expressing high levels of cell surface TNF were generated following four sequential rounds of FACS staining and sorting with an anti-TNF monoclonal antibody. Etanercept and infliximab were equivalent in their ability to bind and neutralize the cell surface TNF as measured by FACS analysis and bioassay, respectively. As previously reported, infliximab was able to mediate complement-dependent killing of the TNF-expressing cells (60% lysis at 0.5 mg/mL). In contrast, etanercept was not able to mediate complement-dependent killing of the TNF-expressing cells (0% lysis at 1.0 mg/mL). The data highlight a unique difference between these TNF antagonists and suggests that etanercept and infliximab may have different mechanisms of action *in vivo*.

Disclosure: work reported in this abstract was supported by: Immunex Corporation, Seattle, Washington, supported work reported in this abstract.

1. Reference not provided.
2. Scallon et al. Cytokine 7:251-259, 1995. [Context Link]

Section Description

Hall C Abstracts # 115 - 210

ACR Poster Session A

Health Services and Outcomes I

Sunday, November 14, 1999, 8:00 AM-9:30 AM

ACR Poster Session A

Cytokines and Mediators I

Sunday, November 14, 1999, 8:00 AM-9:30 AM

Accession Number: 00000889-199909001-00197

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Monoclonal Antibodies to Human Tumor Necrosis Factors Alpha and Beta: Application for Affinity Purification, Immunoassays, and as Structural Probes

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ABSTRACT

Monoclonal antibodies were produced against two structurally related tumor necrosis factors (TNFs), TNF- α (previously called tumor necrosis factor) and TNF- β (previously called lymphotoxin). The potential of these antibodies for the purification of TNFs, the development of specific immunoassays, and for defining the antigenic and functional domains of these cytokines was investigated. None of the monoclonal antibodies cross-reacted with both TNF- α and TNF- β , or reacted with synthetic peptides which represented several of the regions of homology between these cytokines. Neutralizing monoclonal antibodies were utilized as immunoadsorbents to purify recombinant TNF- α and TNF- β from *E. coli* lysates. TNFs purified by this method were greater than 98 percent pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and exhibited specific activities that were the same as TNFs isolated from natural sources using conventional chromatographic techniques. In addition, specific ELISA assays were developed that could detect less than 1 ng/ml of TNF- α or TNF- β , and in contrast to bioassays, could discriminate between these related cytokines.

INTRODUCTION

Tumor necrosis factor (TNF) and lymphotoxin (LT) were originally described as activities that were cytostatic or cytotoxic for a number of tumor cell lines *in vitro*, but had no apparent harmful effects on normal cells in culture (1-8). These two factors had similar biological activities and were initially distinguished from each other solely on the basis of cellular source and induction conditions. Tumor necrosis factor was described as an activity that could cause the necrosis of transplanted tumors in mice (1-4). It was first discovered in the serum of mice and rabbits after they had been injected with bacillus Calmette-Guerin and endotoxin (1,4). TNF activity was also found in the media of activated mononuclear cell cultures (2-4), and was thought to be produced by macrophages (1-4). Lymphotoxin was the name given to a factor with anti-tumor cell activity that was found in the media of activated lymphocyte cultures (5-8). These factors were produced in very small amounts by normal cells, which made their purification and characterization

difficult. Recently, we have reported the purification of two cytotoxic factors derived from continuous tumor cell lines, TNF- α (previously called TNF) produced by a human promyelocytic leukemia cell line (HL60) and TNF- β (previously called LT), produced by a human B lymphoblastoid cell line (RPMI 1788) (9-11). Most of the amino acid sequences of both molecules were determined (10,11) and were found to be different but related. Using this amino acid sequence information, recombinant DNA techniques were utilized to clone the cDNAs for TNF- α and TNF- β and to express biologically active molecules in *E. coli* (12,13). A comparison of the complete amino acid sequences of these two proteins shows that 28 percent of the residues are identical and 51 percent are homologous, representing conservative amino acid changes (11,13). In addition, both cytokines have been shown to bind to the same cell surface receptor (14), and their genes have been found to be closely linked on chromosome six (15). Furthermore, it has been shown that both TNF- α and TNF- β can cause necrosis of Meth A tumors *in vivo* (12,13), a property which was originally used to name TNF. Due to these structural and functional similarities, TNF and LT have been renamed TNF- α and TNF- β (16,17), a nomenclature analogous to that used for interferons.

TNF- α and TNF- β cannot be distinguished on the basis of cytolytic activity in a standard L-929 cell lysis bioassay (1,6). In addition, the purification procedures used for TNF- α and TNF- β are complicated multi-column processes (9-11), making it cumbersome to purify TNFs from natural or recombinant sources. We, therefore, produced monoclonal antibodies against TNF- α and TNF- β in order to develop simple immunoaffinity purification procedures for these proteins, to discriminate between TNF- α and TNF- β by selective neutralization and quantitative immunoassays, and for use as structural probes to define the regions of the molecules responsible for their cytolytic activity.

In this report, we describe the isolation of both neutralizing and non-neutralizing monoclonal antibodies to TNF- α and TNF- β . These antibodies were used to purify recombinant TNFs, yielding homogenous biologically active proteins. Various monoclonal and polyclonal antibodies were used to develop specific ELISA assays that were about as sensitive as a standard L-929 cytolytic assay (11) but were faster and could discriminate between TNF- α and TNF- β . Binding studies and neutralization assays showed that none of the monoclonal antibodies reacted with both TNF- α and TNF- β . In addition, none of the antibodies showed any reactivity with synthetic peptides which represented some of the regions of homology between these cytokines. Several non-neutralizing antibodies were identified which reacted with a synthetic peptide that represents the amino-terminal region of TNF- β . These antibodies were also specific for a 171 amino acid form of TNF- β and did not react with a biologically active truncated form which lacks 23 N-terminal amino acids.

MATERIALS AND METHODS

Immunization Protocols

BALB/c mice were immunized with a combination of natural human TNF- α , purified from HL-60 cell line supernatants (11) and *E. coli* derived human recombinant TNF- α (13), purified using a similar protocol. Mice received an initial injection of natural TNF- α (1.7 μ g) emulsified in CFA and administered IP and SC. The animals were then boosted SC and IM at seven-day intervals with TNF- α adsorbed onto alum (0.1 ml of Al(OH)₃, 1.74 percent w/v in PBS), once with natural TNF- α (1.7 μ g) and once with recombinant TNF- α (10 μ g). Serum collected after the second boost was positive for neutralizing antibodies. Two weeks later, the animals were further boosted with recombinant TNF- α , 50 μ g injected IV and 125 μ g emulsified in IFA and injected SC. Nine weeks after the initial immunization and prior to fusion, the mouse which produced the highest titer of antibodies was injected with a large dose of recombinant TNF- α (1.4 mg) using a protocol that has been reported to

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result in the production of high numbers of positive hybridomas (18) and yield high affinity antibodies (19).

BALB/c mice were immunized with TNF- β purified from RPMI 1788 cell line supernatants (9). The immunization protocol used for TNF- β was significantly different from that used for TNF- α . Several attempts to immunize mice with TNF- β yielded low titer antibodies that failed to neutralize biological activity. In an attempt to enhance its immunogenicity, TNF- β was polymerized by treatment with glutaraldehyde (1 M). Mice received an initial injection of polymerized TNF- β (poly-TNF- β) (50 μ g) emulsified in IFA and administered SC. Twice, at seven-day intervals, the animals were boosted IM and IP with poly-TNF- β (100 μ g) emulsified in IFA. Serum collected after the second boost was positive for antibodies by ELISA, but failed to neutralize biological activity. One week later, the mice were further boosted SC with poly-TNF- β (100 μ g) adsorbed on alum and IV with untreated TNF- β (10 μ g). Serum collected after the third boost was positive for neutralizing antibodies. Eight weeks after the initial immunization, the mouse that produced the highest titer antibodies was boosted IV with untreated TNF- β (15 μ g) and its splenocytes were harvested for fusion four days later.

Polyclonal antisera was obtained by immunizing NZW rabbits ID, at several sites, with recombinant TNF- α (1 mg) or TNF- β (1 mg) emulsified with CFA. At two-week intervals, the animals received booster injections SC, at several sites, with TNF- α (200 μ g) or TNF- β (200 μ g) emulsified with IFA. Ten days after booster injections, sera were collected and evaluated by ELISA and for neutralization of biological activity. The resulting pooled antisera had a neutralizing titer of approximately 900×10^3 units and 45×10^3 units of TNF- α and TNF- β neutralized per ml of serum, respectively.

Fusion and Cell Culture

Splenocytes were harvested from hyperimmune mice and were fused with NP3X63AG8.653 murine myeloma cells as previously described (20). The hybridomas were cultured in HAT medium (DMEM; glutamine, 2.9 g/l; 2-mercaptoethanol, 50 μ M; aminopterin, 10 μ M; hypoxanthine, 100 μ M; thymidine, 31.4 μ M supplemented with 10 percent NCTC 135 medium and 20 percent FBS), in five 96-well tissue culture plates which contained 2×10^4 BALB/c peritoneal macrophage feeder cells/well. The hybridomas were selected in HAT medium for fourteen days, and grown in HT medium (same as HAT but lacking aminopterin) thereafter. The cultures were screened by ELISA about fourteen days after fusion and positive cultures were cloned by limiting dilution in 96 well plates, using BALB/c thymocytes (10^6 /well) as feeder cells.

ELISA Screening Assay

Micro-ELISA plates (Dynatech Immulon II round bottom) were coated for 16 hours at 4°C with purified recombinant TNF- α (170 ng/well) or recombinant TNF- β (100 ng/well) in 50 mM sodium carbonate buffer, pH 9.6. The unreacted protein binding sites on the wells were blocked by incubating for 30 minutes at 22°C with 1 percent gelatin (w/v) in 150 mM NaCl, 50 mM tris, 2 mM EDTA pH 7.4 (TBS) (150 μ l/well). The plates were then washed once with PBS containing 0.05 percent Tween 20 (PBS-Tween). Hybridoma culture supernatants serially diluted with TBS containing 5 mg/ml BSA and 0.05 percent Tween 20 (sample buffer), were added to antigen coated wells and allowed to bind for 2 hours at 22°C. After five rinses with PBS-Tween, horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel) (100 μ l/well) diluted 1:10,000 with sample buffer was added and allowed to bind for 1 hour at 22°C. The plates were rinsed five times with PBS-Tween and incubated 30 minutes at 22°C with 0.1 mg/ml o-phenylenediamine in 0.1 M phosphate-citrate buffer, pH 5.0 containing 0.012 percent H₂O₂ (100 μ l/well). The reaction was stopped by the addition of 2.5 N sulfuric acid (50 μ l/well) and the absorbance of each well was determined using a Titertek Multiscan autoreader. The titers were expressed as the reciprocal of the dilution required to achieve 50 percent of the maximum absorbance at 492 nm.

Neutralization of TNF Cytotoxicity

Supernatants derived from growing hybridomas or purified monoclonal antibodies were serially diluted with RPMI 1640, 50 U/ml penicillin, and 50 µg/ml streptomycin supplemented with 10 percent FBS. These test samples were incubated with an equal volume of the same medium containing approximately 200 U TNF-α or TNF-β for 16 hours at 37°C. The samples were then assayed for lysis of murine L-929 cells (11). These incubation conditions did not significantly reduce TNF-α or TNF-β biological activity in the absence of added antibody.

Monoclonal Antibody Isotyping

The isotype of the various anti-TNF monoclonal antibodies was determined by ELISA. Micro ELISA plates were coated for 16 hours at 4°C with isotype-specific rabbit antiserum (Miles) (100 µl/well diluted 1/1000 in 50 mM sodium carbonate buffer pH 9.6). Undiluted hybridoma culture supernatants were incubated in the coated wells for 2 hours at 22°C. The washing and further processing were done as described above for the ELISA screening assay.

Screening of Monoclonal Antibodies for Use in Immunoaffinity Chromatography by ELISA

The binding of anti-TNF monoclonal antibodies to antigen under various pH conditions and chaotropic salt concentrations was measured by ELISA. Test antibodies were allowed to bind to TNF-α or TNF-β coated micro ELISA wells for two hours at 22°C. Thereafter, the wells were washed five times with PBS containing 0.05 percent Tween 20. The wells were then treated for 1 hour at 22°C with either PBS, 0.15 M NaCl, 50 mM sodium acetate pH 4.0-7.0, 0.15 M NaCl, 50 mM sodium borate pH 7.5-11.0, 1.25 M KSCN, 20 mM tris pH 7.4; or 3.5 M KSCN, 20 mM tris pH 7.4. The wells were then washed five times with Tween 20 and further processed as described above for the ELISA screening assay. The absorbance values of treated wells were compared with PBS control wells and any signal less than 50 percent of the PBS well was considered to be negative for binding.

Purification of Monoclonal Antibodies

Hybridoma cells were grown as ascites tumors in pristane primed BALB/c mice as previously described (21). Monoclonal antibodies were then purified from ascites fluid by DEAE Affigel Blue (BioRad) chromatography (22). Monoclonal antibodies were also purified from tissue culture supernatants using a similar procedure. Hybridoma cells were cultured in a modified low serum medium containing: high glucose DMEM/F12 (1:1); Hepes, 10 mM; β-mercaptoethanol, 50 µM; ethanolamine, 20 µM; glutamine, 2.9 g/l; insulin, 5 mg/l and supplemented with 2.5 percent FBS (23). Cells were grown to a density of about 10⁶ cells/ml in spinner flasks, the medium was harvested by filtration through a 3 µm filter (Pall) and concentrated 10-fold by ultrafiltration using a PM10 membrane (Amicon). The IgG fraction was isolated by precipitation with 50 percent saturated ammonium sulfate, and then dialyzed against 20 mM tris pH 7.2. Thereafter, the IgG was purified by DEAE Affigel Blue chromatography. Purified antibodies were concentrated about 10-fold by ultrafiltration using a PM10 membrane to a final concentration of about 10 mg/ml.

Preparation of Immunoabsorbent Sepharose

Purified monoclonal antibodies were extensively dialyzed against PBS and adjusted to a final concentration of 2 mg/ml. Cyanogen bromide activated Sepharose 4B (Pharmacia) was soaked in 1 mM HCl for 10 min and was then washed on a buchner funnel with 1 mM HCl (200 ml per gram dry gel). The washed gel

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was rinsed with 1 gel volume of PBS and was mixed with the solution of purified monoclonal antibody at a ratio of 1 ml packed gel/ml antibody solution. The gel was allowed to react for 16 hours at 4°C with gentle agitation. The unreacted groups on the gel were blocked by incubating with an equal volume of 0.9 M ethanolamine-HCl, pH 8.0, for 2 hours at 4°C. The gel was then washed first with 5 volumes of PBS, and then with 5 volumes of 0.15 M NaCl, 0.1 M acetic acid and then with 10 volumes of 0.15 M NaCl, 0.05 M tris, 2 mM EDTA and stored in this buffer at 4°C.

Immunoaffinity Chromatography

All purification steps were carried out at 4°C. Frozen recombinant *E. coli* cells containing TNF- α or TNF- β expression plasmids (12,13) were suspended in 10 volumes of lysis buffer (0.2 M NaCl, 0.1 M tris, 50 mM EDTA pH 7.5) and then sonicated at full power for 10 min. at 50 percent duty cycle using a Branson W-375 sonicator. The extract was clarified by the addition of polyethyleneimine (pH 8.0) to a final concentration of 0.5 percent (w/v) and then centrifuged at 1100 x g for 15 min. The pellet was discarded, and solid ammonium sulfate was added to the supernatant to 50 percent saturation (TNF- α) or 40 percent saturation (TNF- β). After incubation for 16 hours at 4°C, the precipitate was collected by centrifugation at 10,000 x g for 15 min. The resulting pellet was dissolved in 1 volume (1 ml/gr. cells) of 0.1 M tris, 5 mM EDTA pH 7.5; and filtered using a 0.45 μ m millipore membrane filter. This sample was then loaded onto an immunoabsorbent column pre-equilibrated with TBS at a flow rate of 9.5 cm/hour. After washing the column with 10 volumes of TBS containing 0.05 percent Tween 20 and then with 1 volume TBS at a flow rate of 19 cm/hour, the TNFs were eluted with 0.15 M NaCl, 0.1 M sodium acetate pH 4.5 at a flow rate of 9.5 cm/hour. Fractions were collected into one-tenth volume of 1 M tris pH 8.5 to adjust the pH of the eluate to about 7.8. The columns were washed with about 10 volumes TBS prior to reuse.

Double Sandwich ELISA for TNF- α and TNF- β

Micro-ELISA wells (NUNC) were coated for 16 hours at 4°C with purified anti-TNF- α 1B2-G3 (100 ng/well) or anti-TNF- β 1G11-E4 (200 ng/well) diluted in PBS. Thereafter, the wells were washed once with PBS-Tween and samples, serially diluted with sample buffer, were added and incubated for 2 hours at 22°C. The wells were then washed 5 times with PBS-Tween and incubated for 2 hours at 22°C with rabbit anti-TNF- α or anti-TNF- β (100 μ l), diluted with sample buffer 1:1600 and 1:10,000, respectively. The wells were again washed 5 times with PBS-Tween and incubated for 1 hour at 22°C with goat anti-rabbit HRP conjugate (Cappel) (100 μ l), diluted 1:10,000 with sample buffer. The wells were then washed 5 times and incubated for 30 min. with 0.2 mg/ml o-phenylenediamine in 0.1 M phosphate/citrate pH 5.0 containing 0.012 percent H₂O₂ (100 μ l). The reaction was stopped by the addition of 2.5 N H₂SO₄ (50 μ l/well) and OD₄₉₂ was measured using a Titertek Multiscan autoreader interfaced with an HP computer with Titercalc^R software. Both assays had useful ranges of 0.4-25 ng TNF/ml and their specificity was demonstrated by the lack of a signal generated by 30 μ g/ml of TNF- α in the TNF- β ELISA or by 30 μ g/ml of TNF- β in the TNF- α ELISA.

Gel Electrophoresis and Immunoblots

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (24). Gels were stained with 0.15 percent Coomassie Blue R250 (w/v) in 25 percent isopropanol (v/v), 10 percent acetic acid (v/v), and destained with 10 percent methanol (v/v), 10 percent acetic acid (v/v). To determine purity, gels were analyzed using a scanning laser densitometer (LKB). The binding of antibodies to TNFs separated by SDS-PAGE was assessed by the immunoblot technique. Recombinant TNF- α (2 μ g) or 18 kDa TNF- β (2 μ g) were subjected to electrophoresis on 15 percent acrylamide

SDS-PAGE gels and transferred to nitrocellulose as previously described (25). The reactive sites on the nitrocellulose were blocked by incubating for 30 min with 3 percent gelatin (w/v) in TBS. Test monoclonal antibodies were diluted in 10 µg/ml in 1 percent gelatin (w/v) in TBS and incubated with the nitrocellulose strips for 16 hours at 22°C. The strips were then washed for 15 min each with three changes of PBS-Tween. Bound monoclonal antibodies were detected by incubating for 4 hours with HRP-conjugated anti-murine IgG (Cappel) diluted 1:1000 in 1 percent gelatin. The strips were washed as above and incubated 15 min with 4-chloro-1 naphthol (0.6 mg/ml in 20 percent methanol, 80 percent TBS containing 0.018 percent H₂O₂), rinsed with water and air dried.

Protein Determinations

Protein determinations were performed by the dye binding method of Bradford (26) using bovine IgG as a standard. The protein concentration was also determined by absorbance using the extinction coefficients for pure recombinant TNF-α and 18 kDa recombinant TNF-β of $\epsilon_{280}^{1 \text{ mg/ml}} = 1.6$ and $\epsilon_{280}^{1 \text{ mg/ml}} = 1.68$, respectively.

¹²⁵I-Labeling of TNFs

Highly purified preparations of natural and recombinant TNF-β were iodinated by the chloramine T oxidation method to a specific activity of 70 µci/µg (27,28). Recombinant TNF-α was iodinated using the Bolton-Hunter reagent to a specific activity of 25 µci/µg (29).

Epitope Mapping of Monoclonal Anti-TNF-α Antibodies

Micro ELISA wells (Dynatech removawell) were coated for 16 hours at 4°C with purified anti-TNF-α monoclonal antibodies (200 ng/well) diluted in PBS. The unreacted protein binding sites were blocked by incubating the wells for 30 min with 1 percent gelatin in TBS (150 µl). ¹²⁵I-TNF-α (~10,000 cpm) was incubated with a panel of monoclonal antibodies in uncoated microwells for 16 hours at 22°C. Test antibodies that were preincubated with ¹²⁵I TNF-α were then incubated for 2 hours at 22°C with antibody coated wells. The wells were washed five times with PBS-Tween and the radioactivity bound to each well was measured with a gamma counter.

TNF-α Monoclonal Antibodies Affinity Determination

The affinity of anti-TNF-α monoclonal antibodies was estimated by the method of Muller (30). The radioimmunoassay was performed as follows: The antibodies were diluted in 0.6 ml of TBS, 2 mg/ml gelatin. 0.2 ml ¹²⁵I-TNF (~10,000 cpm), diluted in TBS, 2 mg/ml gelatin containing 1 percent normal mouse serum, was added and allowed to incubate for 5 hours at 22°C. Goat anti-mouse IgG, diluted in PBS containing 5 percent PEG 8000 (w/v) was added and incubated for 1 hour at 22°C. The tubes were centrifuged at 2500 x g for 30 min, decanted, and the pellets were counted in a gamma counter.

Synthetic Peptides

Peptides were synthesized using a solid phase method as previously described (31).

RESULTS

Production of Monoclonal Antibodies

Initial attempts to immunize mice with small amounts of purified natural TNF- α in Freund's adjuvant alone yielded low titers of antibody in both ELISA and neutralization assays. However, boosting these animals with TNF- α adsorbed onto alum resulted in a large increase in both the serum ELISA and neutralization titers. A combination of alum and Freund's adjuvant boosts produced an even higher serum titer of neutralizing antibodies. For four days prior to fusion, a hyperimmune mouse was boosted daily with recombinant TNF- α . This immunization protocol has been shown to yield large numbers of hybridomas secreting high affinity antibodies (18,19). Splenocytes from this animal were fused with myeloma cells as described in "Materials and Methods". The resulting hybridomas were screened for antibody production with recombinant TNF- α by ELISA, and by inhibition of cytolytic activity. Approximately fifty positive cultures were identified and after cloning by limiting dilution, twelve anti-TNF- α clones were isolated and characterized (Table 1).

TABLE 1
Characteristics of Anti-TNF- α Monoclonal Antibodies*

Epitope Binding Group	Clone	Neutralizing	Antigen Release Conditions	Isotype	Affinity L/mole
I	5E3-H3 (TNFB)	+	pH 4, 3.5 M KSCN	K γ 1	10 ⁹
II	1A5-E5 (TNFF)	+	None	K γ 1	10 ¹⁰
II	1B2-G3 (TNFD)	+	None	K γ 1	10 ¹⁰
II	5A10-F2	+	None	K γ 1	5 x 10 ⁹
II	2H10-F12 (TNFE)	+	None	K γ 1	10 ¹⁰
III	3C4-F7	-	pH 4, 1.25 M KSCN, 3.5 M KSCN	K μ	N.D. ^b
IV	2G3-G6	+	1.25 M KSCN, 3.5 M KSCN	K γ 1	N.D.
IV	5G2-G5	+	1.25 M KSCN, 3.5 M KSCN	K γ 1	N.D.
V	3F10-H4 (TNFG)	-	pH 4, 1.25 M KSCN, 3.5 M KSCN	K γ 1	N.D.
N.T. ^a	1E2-G2 (TNFA)	-	pH 4, 1.25 M KSCN, 3.5 M KSCN	K γ 1	N.D.
N.T.	1G2-A2 (TNFC)	+	None	K γ 1	N.D.
N.T.	2B6-G5	-	pH 4, 1.25 M KSCN, 3.5 M KSCN	K γ 1	N.D.

^a N.T., not tested

^b N.D., not determined, preliminary analysis showed the affinity to be less than 10⁹ L/mole

* The procedures used to determine neutralizing ability, antigen release conditions, isotype and affinity are described in "Materials and Methods".

The immunization of several mice with natural TNF- β (25 kDa form) in Freund's adjuvant resulted in the production of low titer antibodies that reacted in ELISA assays, but failed to neutralize TNF- β biological activity. Repeated boosting failed to elicit neutralizing antibodies. In an attempt to increase immunogenicity, TNF- β was polymerized by treatment with glutaraldehyde. Mice immunized with polymerized TNF- β in Freund's adjuvant produced antiserum against TNF- β with high titers detected by ELISA. However, these animals failed to produce neutralizing antibodies until they were further boosted with TNF- β adsorbed on alum. Splenocytes were isolated from a hyperimmune animal and fused with myeloma cells as described in "Materials and Methods". The resulting hybridomas were screened for antibody production with natural TNF- β (25 kDa species) by ELISA and for neutralization of biological activity. Approximately seventy positive cultures were identified, and after cloning by limiting dilution, thirteen ELISA positive clones were isolated and characterized (Table 2).

TABLE 2
Characteristics of Anti-TNF- β Monoclonal Antibodies*

Epitope Binding Group	Clone	Neutralizing	Antigen Release Conditions	Isotype	Binding to recombinant 16 kDa TNF- β
I	2B8-G7 (LTA)	+	pH 4, 1.25 M KSCN, 3.5 M KSCN	K γ ₁	+
I	1G11-E4 (LTB)	+	pH 4, 3.5 M KSCN	K γ ₁	+
II	1C4-G6 (LTC)	-	3.5 M KSCN	K γ ₁	-
II	1F12-G4	-	3.5 M KSCN	K γ ₁	-
II	2E10-H1	-	3.5 M KSCN	K γ ₁	-
II	2F12-F4	-	3.5 M KSCN	K γ ₁	-
II	2G2-F7	-	3.5 M KSCN	K γ ₁	-
II	3B5-H5	-	3.5 M KSCN	K γ ₁	-
II	2A9-E8	-	3.5 M KSCN	K γ ₁	-
III	1D9-E11 (LTD)	-	3.5 M KSCN	K γ ₃	+
III	2D9-B1	-	3.5 M KSCN	K μ	+
III	4E12-H9	-	3.5 M KSCN	K γ ₁	+
III	4H8-G8	-	3.5 M KSCN	K γ ₁	+

* The procedures used to determine neutralizing ability, antigen release conditions, isotype and binding to 16 kDa TNF- β are described in "Materials and Methods".

Characterization of Monoclonal Antibodies

Various monoclonal antibodies were tested for binding to TNF- α and TNF- β by ELISA, and for neutralization of cytolytic activity. Both natural and recombinant TNF- α exhibit an apparent molecular weight of 17 kDa on SDS-PAGE gels (11). In ELISA assays, all of the anti-TNF- α monoclonal antibodies bound equally well to natural and recombinant TNF- α , but did not bind to recombinant murine TNF (32). The failure of monoclonal antibodies produced against human TNF- α to react with TNF from other species has been observed by others (33). Four different clones produced high affinity antibodies which ranged in affinity from 10^{10} to 5×10^9 L/mole (Table 1). Most of the high affinity antibodies could bind to TNF- α at high and low pH and in the presence of 3.5 M KSCN (Table 1). Eight of the twelve monoclonal antibodies could completely neutralize TNF- α activity (Table 1). The best neutralizing monoclonal antibody, clone 109-E11, had a neutralization titer of 2700 units TNF- α neutralized/ μ g of purified IgG. The neutralizing antibodies required an incubation of antigen and antibody for 1 hour at 37°C or 16 hours at 4°C to completely neutralize TNF- α cytolytic activity. None of the TNF- α specific monoclonal or polyclonal antibodies either neutralized or bound to TNF- β , even when tested at concentrations greater than one hundred times the amount required to show neutralization or binding to TNF- α .

In contrast to TNF- α , natural TNF- β , from mitogen stimulated lymphocytes or RPMI 1788 cells, is glycosylated and exists as 25 kDa and 20 kDa forms (9-10). The 20 kDa form is a truncated version of the 25 kDa TNF- β and lacks twenty-three amino acids from the amino terminus (10). When produced in *E. coli*, the 25 kDa and 20 kDa forms of TNF- β lack glycosylation and exhibit molecular weights of 18 kDa and 16 kDa, respectively on SDS-PAGE (12). Natural and recombinant forms of full length and truncated TNF- β are biologically active in cell lysis bioassays (10,12). All of the anti-TNF- β monoclonal antibodies bound to natural 25 kDa and recombinant 18 kDa TNF- β equally well in ELISA assays, indicating that none of the antibodies produced were specific for a carbohydrate moiety. Seven of the thirteen monoclonal antibodies did not bind 16 kDa TNF- β , and were specific for the 18 kDa form, suggesting that these antibodies are specific for the amino terminus of TNF- β (Table 2). Two clones were isolated that produced neutralizing antibodies (Table 2). These antibodies could neutralize the activity of the recombinant 18 kDa as well as recombinant 16 kDa TNF- β . Monoclonal antibody, clone 1G11-E4, had a neutralization titer of 550 units TNF- α neutralized/ μ g of purified IgG. Clone 2B8-G7 was substantially less, and could only neutralize about 40 percent of the TNF- β activity even when used at high concentrations. The neutralization of TNF- β cytotoxic activity was time and temperature dependent, and was maximal after an incubation for 16 hours at 37°C. Only a partial neutralization of biological activity was observed after incubation with TNF- β for 16 hours at 4°C or for 1 hour at 37°C even with an excess of antibody. Anti-TNF- β monoclonal and polyclonal antibodies neither bound nor neutralized TNF- α , even when tested at concentrations greater than one hundred times the amount necessary to show binding or neutralization of TNF- β .

Epitope Mapping of TNF- α and TNF- β Using Monoclonal Antibodies

The number of distinct TNF- α antigenic epitopes, defined by the panel of monoclonal antibodies, was determined by ELISA using an antibody competition method as described in "Materials and Methods". Five epitopes were identified, three of which reacted with neutralizing antibodies. Anti-TNF- α monoclonal antibodies were categorized into five groups based on their binding to these epitopes as shown in Table 1. Members of both groups I and II are neutralizing antibodies, and can bind simultaneously to TNF- α . Group III consists of one non-neutralizing antibody that partially inhibits the binding of neutralizing group I antibodies. Group IV consists of neutralizing antibodies that partially inhibit neutralizing group II antibodies. Finally, group V consists of one antibody that is non-neutralizing, does not affect group I or II binding and can react with denatured TNF- α on western blots. It

is interesting to note that all of the members of an epitope binding group have the same antigen release conditions (Table 1). These conditions are probably determined by the nature of the antigen-antibody interaction and may be similar for all antibodies binding to a given epitope.

Radiolabeled TNF- β did not bind well to several anti-TNF- β monoclonal antibodies in radioimmunoassays or solid phase antibody binding experiments, although greater than 90 percent of the labeled TNF- β could be precipitated by polyclonal antibodies. The lack of binding, probably a result of damage or alteration of the antigen binding sites by the iodination reaction, or because the anti-TNF- β antibodies exhibited low affinity binding, precluded the same epitope binding analysis performed using anti-TNF- α monoclonal antibodies. It can be inferred, however, that the panel of anti-TNF- β monoclonal antibodies bind to at least 3 antigenic epitopes (Table 2). Group I consists of two neutralizing antibodies, group II, antibodies that react with 18 kDa TNF- β but not 16 kDa TNF- β , and group III, antibodies that react with both forms of TNF- β but fail to neutralize biological activity.

Cross-Reactivity of Monoclonal Antibodies with Synthetic Peptides

In an attempt to define the active sites of TNF- α and TNF- β and to precisely define their antigenic domains, we examined several synthetic peptides for their reactivity with anti-TNF monoclonal antibodies. The peptides correspond to some of the regions of homology between TNF- α and TNF- β , and one peptide corresponds to the amino terminal region of TNF- β which has been reported to be non-essential for cytolytic activity (11). The relationship of these peptides to TNF- α and TNF- β is shown in Figure 1. None of the monoclonal antibodies cross-reacted with any of the TNF- α peptides, residues 1-14, 1-30, 15-30 and 39-66 or with the TNF- β peptides, residues 35-57, 82-94 and 139-155. A group of seven different antibodies cross-reacted with a TNF- β peptide, residues 7-19. All of these antibodies were non-neutralizing and were specific for recombinant 18 kDa TNF- β and did not bind to the recombinant 16 kDa form (Table 2), providing further evidence that the amino terminus of TNF- β is not involved in biological activity.

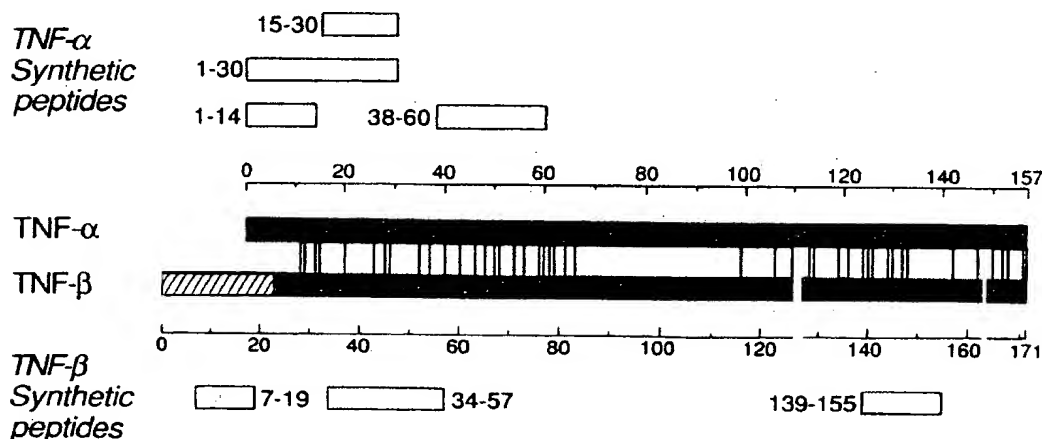


FIGURE 1. Schematic Diagram of TNF- α and TNF- β Proteins Showing Homologous Regions and the Location of Synthetic Peptides. The TNF- α and TNF- β proteins are represented by the horizontal black bars and are numbered by amino acid starting from the amino terminal ends. The hatched region of TNF- β represents the N-terminal 23 amino acids that are missing in the 20 kDa natural and 16 kDa recombinant molecules. The vertical lines between two bars represent homologous amino acids. Synthetic peptides are represented by open boxes and their position corresponds to the sequence as indicated.

Characterization of Monoclonal Antibodies by Immunoblot

Various monoclonal antibodies were examined for their binding to SDS-denatured TNFs using the immunoblot technique (25). A single TNF- α specific antibody and seven anti-TNF- β antibodies were identified that could bind to denatured recombinant TNF- α or TNF- β , respectively (Fig. 2). The anti-TNF- α antibody (3F10-H4) did not cross-react with any of the TNF- α synthetic peptides, was non-neutralizing and bound to a unique epitope of TNF- α that was not cross-reactive with any of the other TNF- α specific antibodies (Table 1). The anti-TNF- β antibodies all shared the same specificity. These antibodies were specific for the amino-terminus of recombinant 18 kDa TNF- β (Table 2), cross-reacted with the TNF- β synthetic peptide, residues 7-19 and were non-neutralizing. These results indicate that many of the anti-TNF monoclonal antibodies, including all of the neutralizing antibodies, probably bind to discontinuous epitopes which are disrupted by treatment with SDS. Because these neutralizing antibodies recognize a precise conformation, it is unlikely that they can react with inactive TNFs.

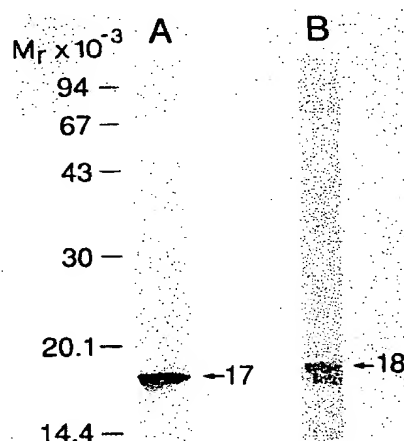


FIGURE 2. TNF Immunoblots Probed with the Monoclonal Antibodies. Recombinant TNF- α reacted with antibody 3F10-H4 (A). Recombinant 18 kDa TNF- β reacted with antibody 1C4-G6 (B). (All of the monoclonal antibodies that were specific for 18 kDa TNF- β gave results identical to 1C4-G6). The positions of the TNF- α and TNF- β bands are shown by the arrows designated 17 and 18, respectively, which indicate the estimated molecular weight in daltons $\times 10^{-3}$.

Immunoaffinity Purification of TNF- α and TNF- β

Two monoclonal antibodies, clone 5E3-H3 and clone 1G11-E4, were chosen for the immunoaffinity purification of TNF- α and TNF- β , respectively. Both antibodies were neutralizing, and their binding to antigen could be reversed using conditions that did not destroy the biological activities of TNF- α or TNF- β . As shown in Figure 3, 5E3-H3 antibodies exhibit reduced affinity for TNF- α below pH 4.5, whereas 1G11-E4 antibodies show reduced binding below pH 6.0 or above pH 9.5. Immunoabsorbents were prepared by coupling purified monoclonal antibodies to cyanogen bromide activated Sepharose as described in "Materials and Methods". About 90 percent of the antibodies were bound to the

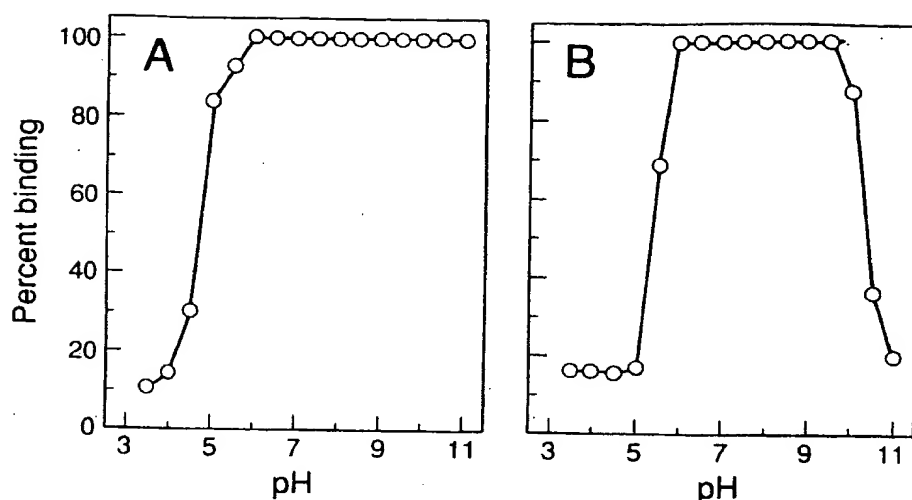


FIGURE 3. The Effect of pH on the Binding of 5E3-H3 Antibodies to TNF- α (A) and 1G11-E4 Antibodies to TNF- β (B). The binding was measured by ELISA as described in "Materials and Methods". Percent binding is relative to the binding at pH 7.4 which represents 100 percent.

gel and the immobilized antibodies retained 50 percent of their theoretical antigen binding capacity.

The anti-TNF- α monoclonal antibody column provided a substantial purification of recombinant TNF- α in a single chromatographic step. A typical purification profile is shown in Figure 4(A). Prior to immunoaffinity chromatography, TNF- α was partially purified from *E. coli* cell lysates by

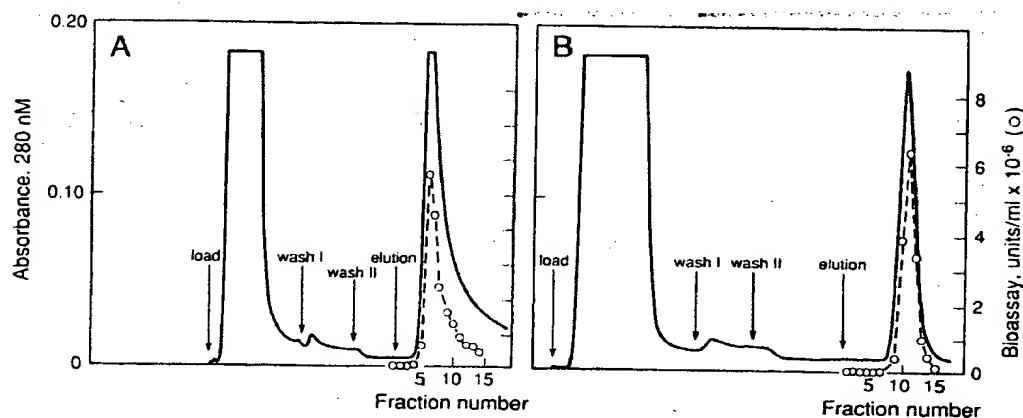


FIGURE 4. Immunoaffinity Column Chromatographic Profiles for TNF- α (A) and TNF- β (B). A column of anti-TNF- α (antibody 5E3-H3) Sepharose (4.5 x 1.6 cm) was loaded at a flow rate of 9.5 cm/hr with 0.5 ml of a 50 percent saturated ammonium sulfate fraction of an *E. coli* lysate containing recombinant TNF- α . The column was washed at a flow rate of 19 cm/hr with TBS containing 0.05 percent Tween 20 (wash I) and then TBS alone (wash II). Bound TNF- α was eluted at a flow rate of 9.5 cm/hr with 0.15 M NaCl, 50 mM Na acetate, pH 4.5, and 2 ml fractions were collected into 0.2 ml 1M Tris, pH 8.5. A column of anti-TNF- β (antibody 1G11-E4) Sepharose (9.3 x 1.6 cm) was loaded with 5 ml of a 40 percent saturated ammonium sulfate fraction of an *E. coli* lysate containing recombinant TNF- β . The washes and elution conditions are the same as those used for TNF- α . The activity of the purified TNF- α and TNF- β fractions was measured using a standard L-929 cell lysis assay.

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precipitation with ammonium sulfate and the reconstituted precipitate was filtered. This pretreatment prevented the accumulation of a colored material on the immunoaffinity column and prevented clogging. Dialysis of the dissolved ammonium sulfate precipitate was not necessary. When this crude bacterial extract fraction was loaded on the antibody column, most of the *E. coli* proteins passed through, and all of the TNF- α activity was retained. The addition of 0.05 percent Tween 20 in the column washes was necessary to remove minor contaminants that adsorbed non-specifically to the column matrix. The bound TNF- α was eluted from the column using a pH 4.5 buffer. We found that TNF- α and TNF- β are not stable at pH 4.5 for more than a few hours at 22°C, therefore, the eluted fractions were collected into 1/10 volume of 1M Tris pH 8.5 to adjust the pH of the eluted TNF- α to 7.8 in order to insure the stability of biological activity. Immunoaffinity purified TNF- α was greater than 98 percent pure by SDS-PAGE (Fig. 5) and had a specific activity of 2.9×10^7 cytolytic units/mg (Table 3), which is in good agreement with the value of 3.0×10^7 units/mg obtained for TNF- α purified from natural resources by conventional chromatography (11). In a single chromatographic step, TNF- α could be purified 21-fold from *E. coli* lysates (Table 3).

The anti-TNF- β monoclonal antibody column could be used to purify both forms of recombinant TNF- β . The purification protocol used for TNF- β was essentially the same as that used for TNF- α . A typical chromatographic profile of a TNF- β purification (18 kDa form) is shown in Figure 4(B). Immunoaffinity purified 18 kDa TNF- β was greater than 99 percent pure by SDS-PAGE and had a specific activity of 3.9×10^7 cytolytic units/mg (Table 3), compared with 4.0×10^7 units/mg reported for TNF- β purified from natural sources by conventional chromatography (9). In comparison to a typical TNF- α immunoaffinity purification of 21-fold, TNF- β was purified 285-fold from *E. coli* lysates (Table 3). This difference is due to the relatively higher purity of the TNF- α starting material (Fig. 5). An anti-TNF- β column was used over twenty-five times without any detectable loss in capacity or any change in the purity in the eluted TNF- β . This result indicates that no leaching of immobilized antibody was detected, and that the mild elution conditions used did not damage the antibody binding activity.

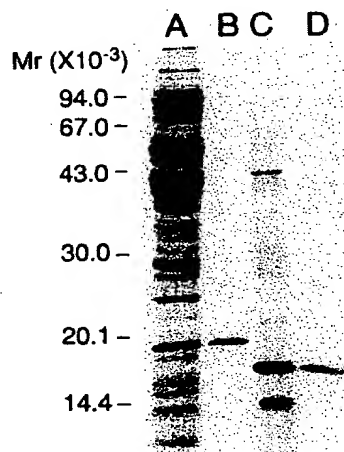


FIGURE 5. Analysis of Immunoaffinity Purified TNFs by SDS-PAGE. 100 μ g of TNF- β immunoaffinity column load (lane A), 2 μ g of immunoaffinity purified TNF β (lane B), 25 μ g of TNF- α immunoaffinity column load (lane C), 2 μ g of immunoaffinity purified TNF- α (lane D). The samples were run on a 12 percent polyacrylamide gel under reducing conditions and stained with Coomassie Blue R-250. By laser densitometry, the purity of immunoaffinity purified TNF- α and TNF- β was determined to be greater than 98 percent and greater than 99 percent, respectively.

TABLE 3
Immunoaffinity Purification of Recombinant TNF- α and TNF- β

Procedure	Protein ^a	Activity ^b	Specific Activity	Yield	Purification
	mg	units ₇ x 10 ⁻⁷	units ₆ /mg x 10 ⁻⁶	Percent	-fold
TNF- α					
E. coli lysate	52	7.1	1.4	100	1
50 percent saturated ammonium sulfate precipitation	18	7.1	3.9	100	2.9
Antibody (TNF β) Sepharose	1.4	4.1	29.0	57.7	21.4
TNF- β					
E. coli lysate	220	3.0	0.14	100	1
40 percent saturated ammonium sulfate precipitation	66.5	3.0	0.45	100	3.3
Antibody (LTB) Sepharose	0.9	3.5	39.0	117	285

^a The protein concentration was determined by the method of Bradford (26).

^b Activity was determined using an L-929 cell cytotoxicity assay (11).

Assay of TNF- α and TNF- β Using Specific ELISA Assays

Specific double sandwich ELISA assays were developed for both TNF- α and TNF- β . Purified neutralizing monoclonal antibodies, anti-TNF- α clone 1B2-G3 or anti-TNF- β clone 1G11-E4, were adsorbed to plastic microELISA wells and utilized to specifically bind TNF in the sample solution. A combination of rabbit polyclonal anti-TNF- α or anti-TNF- β antibodies and HRP conjugated anti-rabbit IgG was then used to detect bound antigen. After incubation with a o-phenylenediamine substrate solution, the absorbance of each well was measured with an automated plate reader and a desktop computer was used for data reduction. Typical standard curves for a TNF- α and a TNF- β ELISA are shown in Figure 6. Both assays had useful ranges of about 0.4 to 25 ng/ml TNF. The specificity of each assay was demonstrated by the lack of any signal generated by 30 μ g/ml of TNF- β in a TNF- α ELISA or by 30 μ g/ml TNF- α in a TNF- β ELISA.

To compare the results obtained from TNF- α and TNF- β ELISA assays with the bioassay, we measured TNF levels in column fractions from typical immunoaffinity purifications of TNF- α and TNF- β using both types of assays (Fig. 7). The ratio of bioactive to immunoreactive TNFs i.e., the specific activity, of all of the TNF- α and TNF- β fractions was relatively constant. The values for the specific activities of TNF- α and TNF- β obtained by this method were $1.7 \pm 0.34 \times 10^7$ units/mg and $3.0 \pm 1.0 \times 10^7$ units/mg, respectively. This result demonstrates that there was no significant difference in the levels of TNF- α and TNF- β measured by ELISA or by bioassay, and that the ELISA assays probably only detect bioactive TNFs.

DISCUSSION

In this report, we described the production, characterization, and application of specific monoclonal antibodies against two very similar

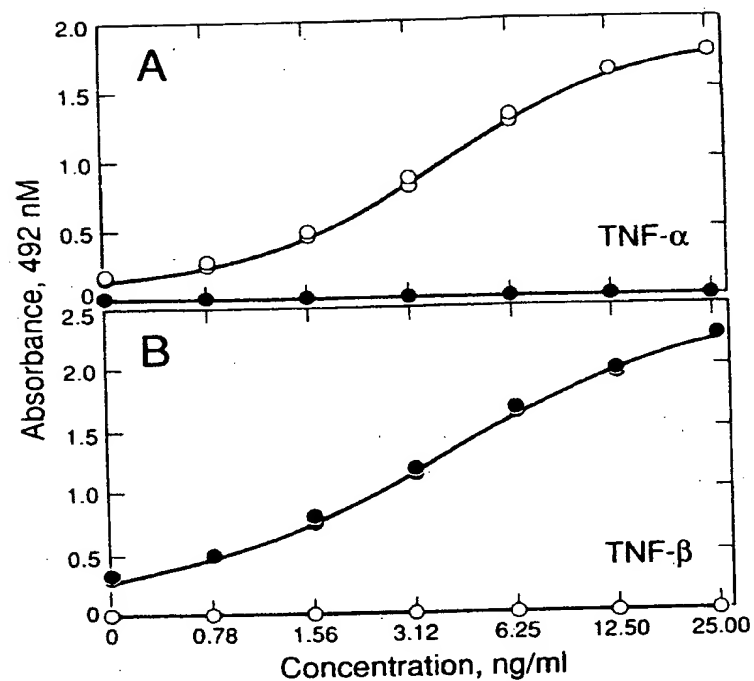


FIGURE 6. Typical Standard Curves for a TNF- α ELISA (A) and a TNF- β ELISA (B). Open circles represent TNF- α and closed circles represent TNF- β . Up to 30 μ g/ml of either TNF- α or TNF- β failed to react in a reciprocal assay.

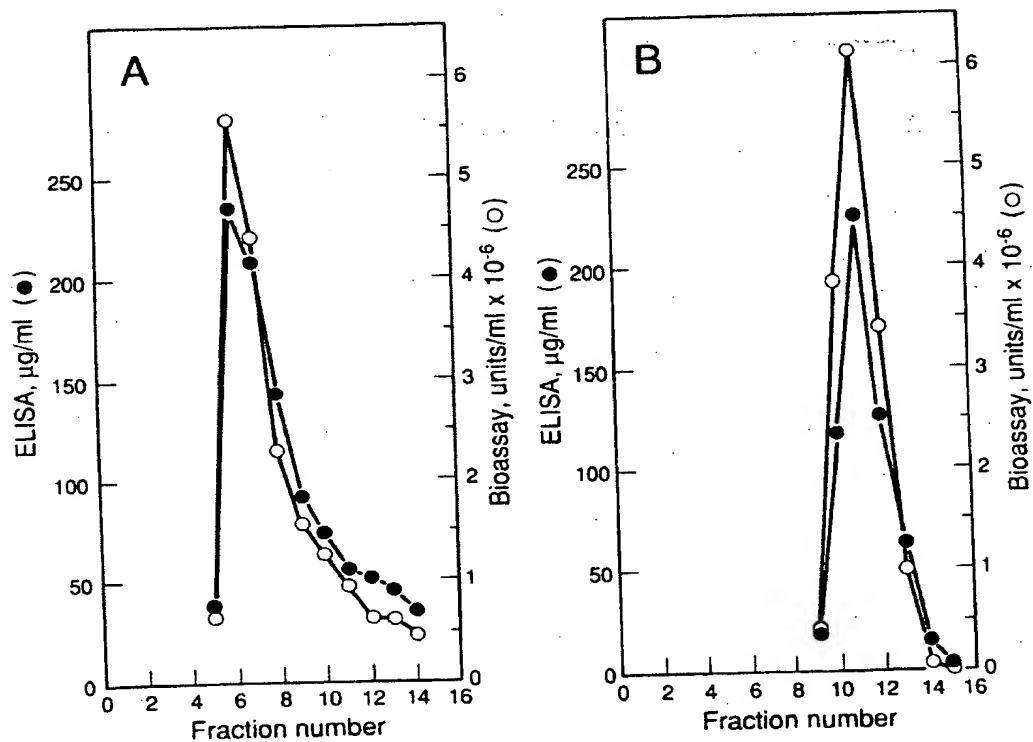


FIGURE 7. Comparison of ELISA Assays with a Standard L-929 Cell Lysis Assay. Fractions from a typical TNF- α (A) or TNF- β (B) immunoaffinity chromatography purification assayed by ELISA and by bioassay.

cytokines, TNF- α and TNF- β . Monoclonal antibodies, as well as rabbit polyclonal antibodies, were highly specific and showed no cross reactivity between these cytolytic proteins. This indicates that despite the fact that these cytokines share substantial amino acid identity, and bind to the same cell surface receptor, their antigenic epitope structures are quite different. We isolated three types of neutralizing anti-TNF- α monoclonal antibodies that bound to distinct antigenic epitopes, including two classes of neutralizing antibodies that could bind simultaneously. These results indicate that TNF- α biological activity is probably not neutralized by antibodies binding to a unique active site, but probably by interfering with the binding of TNF to its cell surface receptor. Non-neutralizing monoclonal antibodies also failed to cross react with both TNF- α and TNF- β , demonstrating that despite their high degree of homology, no antigenic epitopes are shared between these cytokines or that antibodies to shared epitopes are simply rare. We attempted to define the active sites and the antigenic epitopes of TNF- α and TNF- β using synthetic peptides. Most of the antibodies that were isolated were conformation specific, and it was not possible to identify TNF antigenic epitopes by this approach. However, several anti-TNF- β monoclonal antibodies were identified that reacted with amino-terminal sequences of TNF- β that were not required for biological activity. This region may represent the most immunogenic epitope of TNF- β and explain our difficulty in raising neutralizing antibodies in mice.

Various monoclonal antibodies were used to purify milligram quantities of TNF- α and TNF- β , eliminating the need for complicated multi-column procedures. These antibodies were selected because they were neutralizing, and, therefore, likely to bind only to active TNFs, and had reduced affinity for TNFs under acidic conditions that did not destroy cytolytic activity. By using these mild elution conditions, the immunoaffinity columns could be reused many times with no change in performance. A TNF- β specific column was used to purify both recombinant 18 kDa 16 kDa TNF- β . It is likely that this method can be used to purify other engineered variants of recombinant TNFs, eliminating the need for a new purification process for each variant.

TNF monoclonal and polyclonal antibodies were used to develop sensitive sandwich ELISA assays. The TNF ELISA assays were as sensitive as the bioassay, but the ELISA assays could discriminate between TNF- α and TNF- β , which the bioassay cannot, and were faster and easier to perform than the bioassay.

The monoclonal antibodies to both TNF- α and TNF- β described here should prove to be useful in the study of the mechanisms of action of these cytokines. Sensitive ELISA assays can be used to identify and quantitate TNFs and neutralizing antibodies can selectively neutralize TNF cytolytic activity in *in vitro* systems, isolating the effects of TNFs from interferons, lymphokines, and other effector molecules. Recently, several biological activities besides tumor cell killing have been ascribed to tumor necrosis factors. For example, TNF- α has been shown to be anti-parasitic (34) and anti-viral (35,36), and it may play a role in cachexia (37), endotoxic shock (38) and inflammation (39). Both TNF- α and TNF- β can cause bone resorption *in vitro* (40). The observation that myeloma cell lines can produce TNF- β has led to the speculation that TNF- β may be involved in the bone destruction often seen in myeloma patients (40). It is not known whether all of these activities are mediated by TNF binding to the same cell surface receptor. Although, it has been shown that neutralizing TNF- α specific monoclonal antibodies can also inhibit the action of TNF- α on lipoprotein lipase activity in adipocytes. This enzyme is thought to play a key role in cachexia. The availability of monoclonal antibodies against several epitopes of both TNF- α and TNF- β may help to clarify this issue. In addition, the role of TNFs under normal physiological conditions, in tumor killing and under pathological conditions is not yet understood. Specific ELISA assays that can identify and quantitate TNFs will aid in elucidating the role of TNFs in normal and disease states. Finally, neutralizing anti-TNF monoclonal antibodies may be able to block the side effects of TNF *in vivo* providing a specific therapy for TNF mediated disorders.

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ABBREVIATIONS: BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DMEM, Dulbecco's modified Eagle's minimal essential medium; EDTA, ethylenediaminetetraacetate; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HRP, horseradish peroxidase; ID, intradermally; IM, intramuscularly; IP, interperitoneally; IV, intravenously; IFA, incomplete Freund's adjuvant; LT, lymphotoxin; NZW, New Zealand white; PEG, polyethyleneglycol; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SC, subcutaneously; TBS, tris buffered saline; TNF, tumor necrosis factor.

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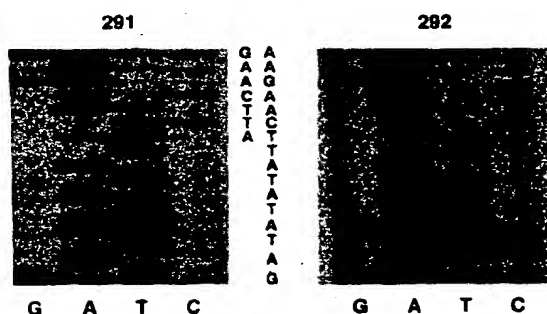


FIG. 3 Direct sequencing of the parents of the affected individual in family BOS22. PCR-amplified DNA was purified and asymmetrically amplified. The resulting single-stranded DNA was sequenced with the CF-17 primer. The sequence for the mother (no. 291) matches that of the father (no. 292) and the published sequence up to the T residue (position 2,566). Beyond this point, the sequence is a mixture of the two alleles owing to an AT insertion. METHODS. DNA amplified with primers CF-8 and CF-17 was electrophoresed on a nondenaturing polyacrylamide gel. The product was excised from the gel and soaked in 100 μ l water for 1 h at 65 $^{\circ}$ C. Eluted DNA (5 μ l) was reamplified with a 50-fold dilution of primer CF-17 for 40 cycles. The amplified DNA was purified with a Centricon 100 column (Amicon) and sequenced with Sequenase (USB) using dITP and 35 S-labelled dATP.

whereas the other carrier sibling had borderline values at 6–7 years of age (49–50 milliequivalents Cl^{-}) that decreased to normal levels (38 milliequivalents Cl^{-}) at 8 years of age. The mother had a normal sweat-test value. The difference in sweat-test values among these individuals could be due to environmental factors or inaccuracies in the sweat test, or reflect additional genetic control over ion transport in the sweat duct. In either case, a more detailed characterization of ion transport and regulation in these individuals should provide insight into these processes.

The CFIns2566 allele is due to the addition of an AT dinucleotide into a short segment (8 bp) of AT dinucleotides. Dinucleotide repeats are hotspots for mutations^{19,20}. Although principally CA repeats have been examined, polymorphic AT repeats have also been characterized. A search of the primate sequences in the computerized database GenBank revealed >50 sequences containing AT repeats that were of 22 bp or more (M.D., data not shown). The mechanism for generating new alleles at these loci is not understood, but could involve unequal crossing-over or errors in replication. Examination of new alleles at other tandemly repeated loci, however, indicates that more complex mechanisms could be involved²¹.

The identification of all of the mutations that cause CF is essential for complete detection and diagnosis of the disease. Although the CFIns2566 allele seems to be rare, the identification of this mutation provides some important insights. First, all of the CF mutations do not lie in the same exon, implying that complete detection will probably require examination of several regions of the gene. Second, frame-shift and other null mutations might not be uncommon. The most likely explanation for the failure so far to find such mutations in the CF gene is that individuals homozygous for the loss of gene function do not survive. If carriers for termination mutants are healthy, there would be no selection against such alleles; these alleles would only appear in CF individuals, however, when balanced by a less severe allele. Frame-shift mutations could occur in virtually any region of the gene, making CF diagnosis difficult.

The continued identification of mutations in the CFTR locus is expected to help elucidate which regions of the CFTR are functionally important. Also, examination of the effects of these mutations in the allelic combinations in which they naturally occur should greatly increase our understanding of the function of the CFTR gene and its role in disease. \square

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Biological properties of a CD4 immunoadhesin

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MOLECULAR fusions of CD4, the receptor for human immunodeficiency virus (HIV; refs 1–4), with immunoglobulin (termed CD4 immunoadhesins) possess both the gp120-binding and HIV-blocking properties of recombinant soluble CD4, and certain properties of IgG, notably long plasma half-life and Fc receptor binding^{5,6}. Here we show that a CD4 immunoadhesin can mediate antibody-dependent cell-mediated cytotoxicity (ADCC) towards HIV-infected cells, although, unlike natural anti-gp120 antibodies, it does not allow ADCC towards uninfected CD4-expressing cells that have bound soluble gp120 to the CD4 on their surface. In addition, CD4 immunoadhesin, like natural IgG molecules, is efficiently transferred across the placenta of a primate. These observations have implications for the therapeutic application of CD4 immunoadhesins, particularly in the area of perinatal transmission of HIV infection.

We have previously described CD4 immunoadhesins containing the first two immunoglobulin-like domains of CD4 joined to the entire constant region of human IgG1 heavy chain⁵. As the presence of light chain was found to be unnecessary for secretion of dimeric molecules⁵, we constructed additional derivatives lacking the CH1 domain of the IgG1 heavy chain (Fig. 1). The gp120-binding and Fc receptor-binding properties and the improved half-life characteristics of this molecule were comparable to the CD4 immunoadhesin containing the CH1 domain (not shown).

As CD4 immunoadhesin binds Fc receptors, we examined

TABLE 1 Placental transfer of rCD4 and CD4-IgG in pregnant rhesus monkeys

Rhesus monkey	Protein	Concentration (ng ml ⁻¹) in maternal serum		Concentration (ng ml ⁻¹) in newborn serum	Infant/maternal concentration ratio
		Mean	Range		
1	CD4-IgG	489	(276-673)	15.2	3.1%
2	CD4-IgG	217	(155-301)	7.6	3.5%
3	rCD4	682	(360-820)	1.1	0.16%
4	rCD4	437	(205-504)	<0.8	<0.18%

Four pregnant rhesus monkeys at 150-160 days gestation (normal gestation period 160-170 days) received a loading dose of CD4 immunoadhesin (CD4-IgG) or rCD4 by rapid intravenous injection followed by continuous infusion for 24 h; the infants were delivered by caesarian section. Blood was obtained from the mother after 1 min and after 4, 8, 12, 16, 20 and 24 h of infusion and from the infant and cord blood at the time of delivery. For maternal dosing and blood sampling, catheters were placed into a femoral vein and artery under general anaesthesia 24 h before the start of the study. After catheterization the animals were placed into jackets, and no additional anaesthesia was given. Animals received the loading dose of drug as an intravenous bolus into the femoral vein catheter over 5 s followed by saline flush to clear the catheter of drug. The infusion was started immediately thereafter. For CD4 immunoadhesin, a 0.135 mg kg⁻¹ loading dose was given, followed by 1.12 mg kg⁻¹ over 24 h; for rCD4, a 0.135 mg kg⁻¹ loading dose was given, followed by 28 mg kg⁻¹ over 24 h. Serum concentrations of each protein were determined by double antibody enzyme-linked immunosorbent assays (ELISA) each using monoclonal antibody Leu3a (Becton-Dickinson). As this antibody recognizes the gp120 binding domain of CD4, the assays thus detect CD4-containing molecules still capable of binding gp120. To measure rCD4 concentration, Leu3a in 0.05 M carbonate buffer, pH 9.6, was used to coat 96-well microtitre plates overnight at 4 °C. After three washes with PBS containing 0.05% Tween 20 (PBS-Tween), plates were blocked for 1 h at room temperature with ELISA diluent (PBS containing 0.5% BSA, 0.05% Tween 20 and 0.01% thimerosal). rCD4 standards and samples diluted in rhesus serum were incubated for 2 h, and plates were washed again with PBS-Tween. For detection of rCD4, monoclonal antibody OKT4 (Ortho) was conjugated to horseradish peroxidase (HRP, Boehringer Mannheim) by the periodate method¹⁹. After appropriate dilution in ELISA diluent, the conjugated antibody was incubated for 1 h at ambient temperature. Orthophenylene diamine dihydrochloride (Sigma), 2.2 mM in 0.05 M sodium phosphate/0.1 M citrate buffer, pH 5.0, containing 0.01% H₂O₂, was used as a substrate for 20-30 min at room temperature. Reactions were stopped with 4.5 N H₂SO₄ and plates were read at 492 nm. Data were reduced using a four-parameter curve-fitting program²⁰. The range for this assay was 0.8 to 25 ng ml⁻¹. For the measurement of CD4 immunoadhesin, the same procedure was used, except that Leu3a was conjugated to HRP and used for detection; monoclonal antibody L104.5 (provided by B. Fendly, Genentech), which recognizes domain 2 of rCD4, was used for antigen capture. The range for this assay was 0.19 to 12.0 ng ml⁻¹.

whether it could mediate ADCC towards HIV-infected cells by human peripheral blood mononuclear cells. Indeed, CD4 immunoadhesin mediates ADCC towards HIV-infected, but not uninfected, CEM human T-lymphoblastoid cells in a dose-dependent manner (Fig. 2a and b). Soluble recombinant (rCD4) does not mediate ADCC (not shown), but can inhibit cell lysis mediated by CD4 immunoadhesin (Fig. 2a), demonstrating that specific binding to gp120 by CD4 immunoadhesin is essential.

It has been suggested that ADCC in AIDS patients may be a mechanism of pathogenesis rather than protection⁷, as soluble gp120, by binding to healthy CD4-expressing 'bystander' cells, can make such cells targets for ADCC, mediated by the anti-gp120 antibodies found in HIV-infected individuals. In contrast to natural anti-gp120 antibodies, CD4 immunoadhesin does not

mediate killing of uninfected CEM cells preincubated with soluble gp120 (Fig. 2b). A likely explanation is that CD4 immunoadhesin, unlike natural anti-gp120 antibodies, cannot bind gp120 already bound to cell-surface CD4, because soluble gp120 is thought to have only one CD4-binding site.

An increasing number of paediatric AIDS patients are infected *in utero* by transmission from the mother⁸. As natural IgG molecules are selectively transported across the placenta of primates in an Fc receptor-dependent manner, we tested whether CD4 immunoadhesin shared this property. Pregnant rhesus monkeys near to term were given a bolus dose of either rCD4 or CD4 immunoadhesin, then were continuously infused to a relatively constant concentration for 24 h before delivery by caesarian section. Serum concentrations were determined by

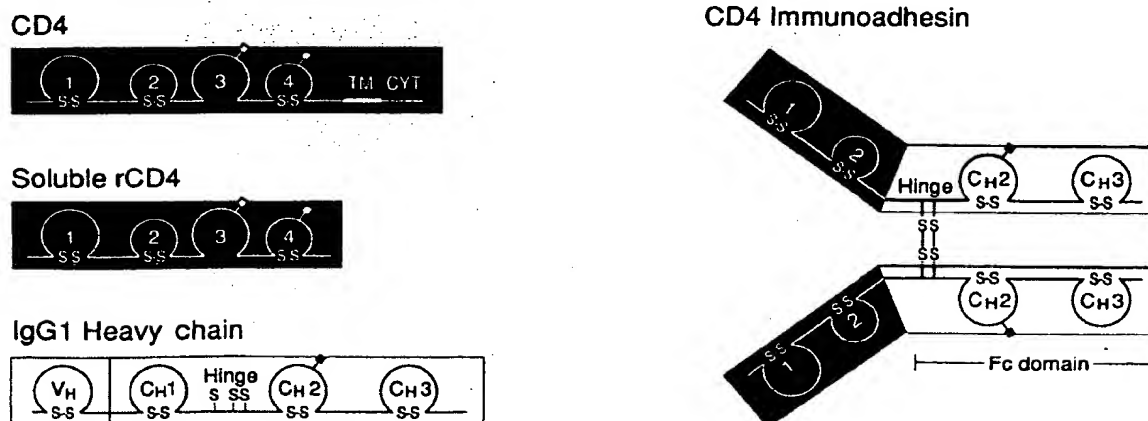


FIG. 1 Structure of CD4 immunoadhesin, soluble rCD4 and the parent human CD4 and IgG1 heavy chain molecules. CD4- and IgG1-derived sequences are indicated by shaded and unshaded regions, respectively. The immunoglobulin-like domains of CD4 are numbered 1-4; TM and CYT refer to the transmembrane and cytoplasmic domains. Soluble rCD4 is truncated after proline 368 of the mature CD4 polypeptide¹⁴. The variable (VH) and constant (CH1, hinge, CH2, and CH3) regions of IgG1 heavy chain are shown. Disulphide bonds are indicated by S-S. CD4 immunoadhesin consists of

residues 1-180 of the mature CD4 protein fused to IgG1 sequences beginning at aspartic acid 216 (taking amino acid 114 as the first residue of the heavy chain constant region¹⁵) which is the first residue in the IgG1 hinge after the cysteine residue involved in heavy-light chain bonding. The CD4 immunoadhesin shown, which lacks a CH1 domain, was derived from a CH1-containing CD4 immunoadhesin⁵ by oligonucleotide-directed deletion mutagenesis¹⁸, expressed in Chinese hamster ovary cells and purified to >99% purity using protein A-Sepharose chromatography as described⁵.

immunoassay at various times in the mother and in the newborn within 5 min of birth. The concentration of CD4 immunoadhesin in fetal serum was $\geq 3\%$ of the maternal level after 24 h (Table 1), indicating a significant rate of placental transfer. By contrast, rCD4 did not accumulate in the fetal serum to a significant extent. This is most probably due to lack of active transport across the placental barrier, although it is possible that transfer would not be detected owing to the shorter half-life of rCD4 (ref. 5).

Although the rate at which a protein appears in the fetal circulation cannot be directly translated into a rate of placental transfer, because the rate of degradation of the protein in the fetus is unknown, comparisons can be made with the appearance rate of human antibody in classical human experiments. Dancis *et al.*⁹ gave radioiodinated human γ -globulin to women in their third month of pregnancy before abortion of the fetus, and observed a concentration in the fetus that was 2.8% of maternal levels after 18–24 h. Similarly, Gitlin *et al.*¹⁰ gave women who were nearly to term a single intravenous injection of radioiodinated γ -globulin up to 4 weeks before birth and observed an increase in the infant plasma concentration of $\sim 3\%$ of maternal level per day. Thus the rate of appearance of CD4 immunoadhesin in a primate fetus is close to that of normal human IgG in humans.

CD4-based strategies have an important theoretical advantage

over other AIDS therapeutics, as HIV must bind CD4 to be able to infect its cellular target (the T4 cell) specifically. Soluble CD4 derivatives have thus been developed with two objectives: to block gp120-mediated events such as the spread of viral infection, formation of syncytia and binding of gp120 to uninfected 'bystander' cells, and to use CD4 as a targeting agent to direct a cytotoxic agent to HIV-infected cells (for example, CD4-ricin¹¹, CD4-pseudomonas exotoxin¹²). Here we have shown that CD4 immunoadhesin can direct the killing of HIV-infected cells, as well as blocking gp120-mediated events^{3,6}. Significantly, CD4 immunoadhesin, unlike natural anti-gp120 antibodies, cannot kill CD4-expressing bystander cells coated with soluble gp120.

The fetal acquisition of passive immunity in humans is mediated by selective placental transfer of maternal IgG. As CD4 immunoadhesin shares this property, passive immunity to HIV could be established in the fetus by maternal administration, possibly preventing perinatal transmission of infection. The mechanism underlying selective transport of IgG involves binding to Fc receptors on the apical surface of the syncytiotrophoblast, resulting in protected endocytotic transport¹³. The fact that this, and so many other different properties of IgG, can be conferred on CD4 by the addition of an Fc region suggests that such functions could be acquired by any adhesion molecule capable of being linked to Fc in place of the Fab sequences

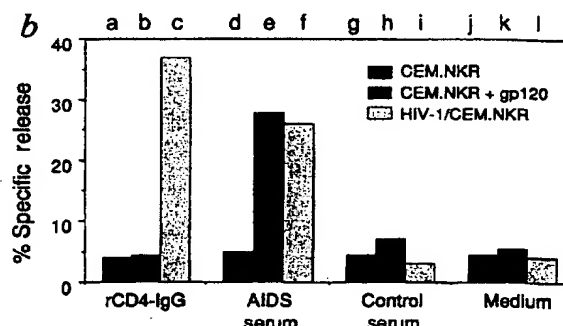
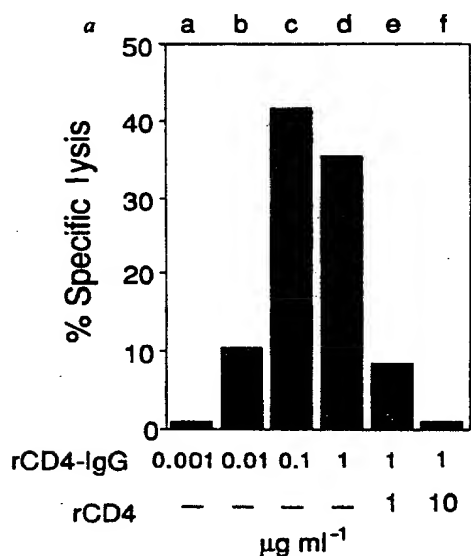


FIG. 2 Antibody-dependent cell-mediated cytotoxicity (ADCC) shown by CD4 immunoadhesin. CEM T-lymphoblastoid target cells were labelled with ^{51}Cr , incubated with CD4 immunoadhesin, rCD4, serum, or control media for 30 min, and mixed with peripheral blood mononuclear cells (PBMC), as effector cells, at an effector-to-target ratio of 50:1. The cell mixtures were incubated for 20 h at 37 °C, and the cell-free supernatant was collected and assayed for ^{51}Cr released from target cells. **a**, Lysis of HIV-1-infected CEM.NKR target cells by effector cells in the presence of CD4 immunoadhesin at 0.001 (lane a), 0.01 (lane b), 0.1 (lane c) and 1.0 $\mu\text{g ml}^{-1}$ (lanes d–f). Also shown is the blocking by rCD4 at 1.0 (lane e) and 10 $\mu\text{g ml}^{-1}$ (lane f) of target cell lysis mediated by 1.0 $\mu\text{g ml}^{-1}$ CD4 immunoadhesin. The level of cell lysis observed with CD4 immunoadhesin was comparable to that mediated by a control AIDS patient serum. rCD4 itself does not mediate target cell lysis at concentrations up to 10 $\mu\text{g ml}^{-1}$. Uninfected CEM.NKR targets were not lysed by effector cells in the presence of CD4 immunoadhesin, AIDS patient serum or normal human serum (see below), but could be lysed in the presence of a rabbit anti-CD4 serum (not shown). **b**, ADCC towards uninfected CEM.NKR target cells (lanes a, d, g and j), uninfected CEM.NKR cells incubated with soluble gp120 (ref. 17) (lanes b, e, h and k) and HIV-1-infected CEM.NKR (lanes c, f, i and l) mediated by CD4 immunoadhesin (lanes a–c), AIDS patient serum at 1/1,000 final dilution (lanes d–f), serum from an uninfected individual at 1/1,000 final dilution (lanes g–i) and complete medium (lanes j–l).

METHODS. The CEM.NKR T-lymphoblastoid cell line, which is resistant to NK-mediated lysis¹⁸, was used for all experiments. HIV-1-infected CEM.NKR cells were produced by inoculating 10^6 CEM.NKR cells with 10^3 TCID₅₀ of HIV-1 II_{IB}. The culture was monitored for infection using reverse transcriptase (RT) activity and HIV-1 specific antibodies for immunofluorescence. After ~ 2 weeks the culture became stable, with $>70\%$ of cells

immunofluorescence-positive, and $>10^6$ c.p.m. ml^{-1} RT activity in the medium. Cells were maintained in RPMI 1640 medium (Gibco) containing 20% fetal bovine serum (MA bioproducts), penicillin, streptomycin and L-glutamine (complete medium). Target cells were labelled by incubation of 10^6 cells with 100 μCi ^{51}Cr in 0.5 ml for 2 h at 37 °C. After two washes, cells were suspended at 2×10^5 per ml in complete medium and 25- μl aliquots (containing 5×10^3 cells) were dispensed to wells of a 96-well plate. For the lysis assay, 25 μl purified recombinant proteins or sera diluted in complete medium, or control medium, were added to each well and incubated for 30 min at room temperature. Assays were carried out in triplicate. Effector PBMCs were prepared from heparinized blood obtained from a healthy, HIV-seronegative donor by centrifugation through Ficoll-Paque (Pharmacia). After two washes in RPMI 1640 the cells were suspended to 5×10^6 cells per ml in complete medium and 50- μl aliquots were added to appropriate wells. The total incubation volume was therefore 100 μl . The concentrations indicated are final concentrations after effector cells were added. Plates were incubated for 16–18 h at 37 °C in 5% CO₂. For analysis of cell lysis, 50- μl samples of supernatant were pipetted from each well, mixed with detergent to inactivate HIV, then mixed with 0.5 ml Protosol (New England Nuclear) and 5 ml Betafluor (National Diagnostics) and analysed by scintillation counting. Maximum lysis, spontaneous lysis and complete medium controls were included in each assay for each target cell, in triplicate. Maximum lysis was obtained by substituting 25 μl of 2% Triton X-100 for the test sample. Spontaneous release wells received 70 μl complete medium instead of effector cells. Complete medium controls received medium instead of the test sample. Percentage specific lysis was calculated using the formula, % specific lysis = (test sample – spontaneous release)/(maximum lysis – spontaneous release).

normally constituting the antigen-binding site of IgG. Therefore, in principle, any such receptor can be given the functional characteristics of an antibody, with the ability to select desirable characteristics at will. □

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Calcium entry through stretch-inactivated ion channels in *mdx* myotubes

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RECENT advances in understanding the molecular basis of human X-linked muscular dystrophies (for a review, see ref. 1) have come from the identification of dystrophin, a cytoskeletal protein associated with the surface membrane²⁻⁴. Although there is little or virtually no dystrophin in affected individuals^{5,6}, it is not known how this causes muscle degeneration. One possibility is that the membrane of dystrophic muscle is weakened and becomes leaky to Ca^{2+} (refs 7-9). In muscle from *mdx* mice, an animal model of the human disease¹⁰, intracellular Ca^{2+} is elevated and associated with a high rate of protein degradation¹¹. The possibility that a lack of dystrophin alters the resting permeability of skeletal muscle to Ca^{2+} prompted us to compare Ca^{2+} -permeable ionic channels in muscle cells from normal and *mdx* mice. We now show that recordings of single-channel activity from *mdx* myotubes are dominated by the presence of Ca^{2+} -permeable mechano-transducing ion channels. Like similar channels in normal skeletal muscle, they are rarely open at rest, but open when the membrane is stretched by applying suction to the electrode¹²⁻¹⁴. Other channels in *mdx* myotubes, however, are often open for extended periods of time at rest and close when suction is applied to the electrode. The results show a novel type of mechano-transducing ion channel in *mdx* myotubes that could provide a pathway for Ca^{2+} to leak into the cell.

We recorded single-channel activity from cell-attached patches on myotubes from normal and *mdx* mice with 110 mM BaCl_2 in the patch electrode. Figure 1a shows a continuous record of single-channel activity recorded ~1 min after the patch electrode formed a seal on the surface of a myotube from normal mouse muscle. At a holding potential of -60 mV, the single-channel

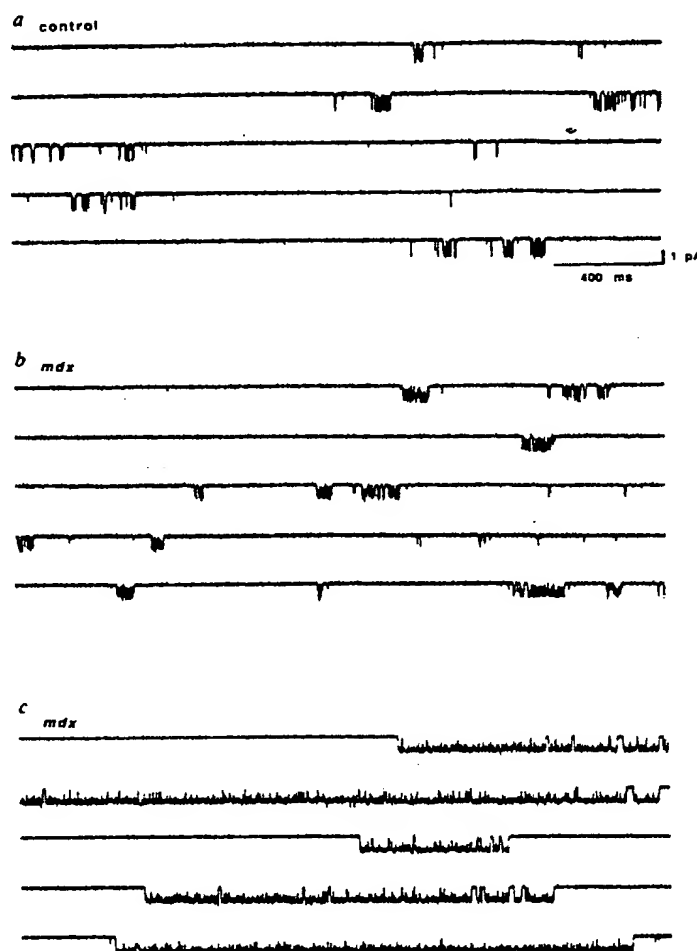


FIG. 1 Channel activity recorded from the surface of myotubes from normal and *mdx* mice with 110 mM BaCl_2 in the patch electrode showing unitary Ba^{2+} currents at a constant holding potential of -60 mV. The traces are sequential and represent a segment of a continuous recording (~10 seconds channel activity). Currents were filtered at 1 kHz with an eight-pole Bessel filter and sampled at 5 kHz. a, Recording from a cell-attached patch on a normal myotube. b, Recording from a cell-attached patch on a *mdx* myotube showing low channel activity. c, Recording from a different *mdx* myotube in which channel activity was high.

METHODS. Myotubes were prepared by dissecting hind-limb or cutaneous-pectoralis muscles from 7-day-old normal C57B control mice or *mdx* mice (Jackson Laboratory) after killing by cervical dislocation. The muscle was minced and incubated for ~15 min at 37 °C in Ca^{2+} - and Mg^{2+} -free Hark's buffer containing 0.125% trypsin. Cells were dissociated by passing through a small-bore pipette and filtered through 100- μm gauze. The suspension was preplated for ~1 h to remove fibroblasts, after which the remaining cells in suspension were plated on gelatin-coated tissue culture dishes at a density of ~5,000 cells per cm^2 in DMEM medium supplemented with 20% FCS and chick embryo extract. Myoblasts began to fuse and form myotubes after ~4-5 days in culture. Recordings were made from myotubes 1-5 days after the first myotubes formed. Recordings of single-channel activity from cell-attached patches were made with a List EPC-7 amplifier as described previously¹⁵. Current signals were recorded on video tape and replayed onto the hard disk of a laboratory computer (PDP 11/73) for later analysis. Patch electrodes were made from borosilicate capillary pipettes (Rochester Scientific) and had resistances of 2-4 M Ω when filled with 110 mM BaCl_2 and immersed in the bath. The bathing solution contained 150 mM potassium aspartate, 5 mM MgCl_2 , 5 mM EGTA, 10 mM glucose and 10 mM HEPES buffer. The pH was adjusted to 6.5 with KOH. An isotonic potassium bathing solution was used to zero the resting potential of the cell. Occasionally, voltage shifts were detected after patch excision which indicated a maximum voltage error of ~10 mV. The bathing solution produced no obvious signs of cell deterioration.

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United States Patent [19]**Capon et al.**[11] **Patent Number:** **5,116,964**[45] **Date of Patent:** **May 26, 1992**[54] **HYBRID IMMUNOGLOBULINS**[75] **Inventors:** Daniel J. Capon, San Mateo;
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Calif.[73] **Assignee:** Genentech, Inc., South San
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435/252.3; 435/320.11; 530/350[58] **Field of Search** 435/69.7, 172.3, 252.3,
435/320; 436/512; 530/350, 387; 536/27[56] **References Cited****FOREIGN PATENT DOCUMENTS**

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[57]

ABSTRACT

Immunoglobulin fusion polypeptides are provided, together with methods for making and using them, and nucleic acids encoding them. These polypeptides are useful as cell surface adhesion molecules and ligands, and are useful in therapeutic or diagnostic compositions and methods.

8 Claims, 18 Drawing Sheets

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FIG. 1-1

GAATTCCAGTGTGCTGGCTTCCTCAGCTGCAGCAGCACACTCCTTTGGGCAAGGACCTGAGACCCCTTGTGCTAAGTC

1 MET ILE PHE PRO TRP LYS CYS
ATG ATA TTT CCA TGG AAA TGT

AAGAGGCTCAATGGGCTGCAGAAGAACTAGAGAAGGACCAAGCAAGCC

10 GLN SER THR GLN ARG ASP LEU TRP ASN ILE PHE LYS LEU TRP GLY TRP THR MET LEU CYS
CAG AGC ACC CAG AGG GAC TTA TGG AAC ATC TTC AAG TTTG TGG GGG TGG ACA ATG CTC TGT

20 SIGNAL SEQUENCE

30 CYS ASP PHE LEU ALA HIS HIS GLY THR TYR CYS TRP THR TYR HIS TYR SER GLU LYS PRO
TGT GAT TTC CTG GCA CAT CAT GGA ACC TAC TGC TGG ACT TAC CAT TAT TCT GAA AAA CCC

40 PROBABLE N-TERMINUS

50 MET ASN TRP GLN ARG ALA ARG ARG PHE CYS ARG ASP ASN TYR THR ASP LEU VAL ALA ILE
ATG AAC TGG CAA AGG GCT AGA AGA TTC TGC CGA GAC AAT TAC ACA GAT TTA GTT GCC ATA

60

70 GLN ASN LYS ALA GLU ILE GLU TYR LEU GLU LYS THR LEU PRO PHE SER ARG SER TYR TYR
CAA AAC AAG GCG GAA ATT GAG TAT CTG GAG AAG ACT CTG CCC TTC AGT CGT TCT TAC TAC

80

90 TRP ILE GLY ILE ARG LYS ILE GLY GLY ILE TRP THR TRP VAL GLY THR ASN LYS SER LEU
TGG ATA GGA ATC CGG AAG ATA GGA GGA ATA TGG ACG TGG GTG GGA ACC AAC AAA TCT CTC

100

110 THR GLU GLU ALA GLU ASN TRP GLY ASP GLY GLU PRO ASN LYS LYS ASN LYS GLU ASP
ACT GAA GAA GCA GAG AAC TGG GGA GAT GGT GAG CCC AAC AAC AAG AAG AAC AAG GAG GAC

120

130 CYS VAL GLU ILE TYR ILE LYS ARG ASN LYS ASP ALA GLY LYS TRP ASN ASP ASP ALA CYS
TGC GTG GAG ATC TAT ATC AAG AGA AAC AAA GAT GCA GGC AAA TGG AAC GAT GAC GCC TGC

140

150 HIS LYS LEU LYS ALA ALA LEU CYS TYR THR ALA SER CYS GLN PRO TRP SER CYS SER GLY
CAC AAA CTA AAG GCA GCC CTC TGT TAC ACA GCT TCT TGC CAG CCC TGG TCA TGC AGT GGC

160

FIG. 1-2

170 HIS GLY GLU CYS VAL GLU ILE ILE ASN ASN HIS THR CYS ASN CYS ASP VAL GLY TYR TYR
 CAT GGA GAA TGT GTA GAA ATC ATC AAT AAT CAC ACC TGC AAC TGT GAT GTG GGG TAC TAT

180
 190 GLY PRO GLN CYS GLN LEU VAL ILE GLN CYS GLU PRO LEU GLU ALA PRO GLU LEU GLY THR
 GGG CCC CAG TGT CAG CTT GTG ATT CAG TGT GAG CCT TTG GAG GCC CCA GAG CTG GGT ACC

200
 210 MET ASP CYS THR HIS PRO PHE GLY ASN PHE SER ASN PHE SER CYS ALA PHE SER CYS
 ATG GAC TGT ACT CAC CCC TTT GGA AAC TTC AGC TCA CAG TGT GCC TTC AGC TGC

220
 230 SER GLU GLY THR ASN LEU THR GLY ILE GLU GLU THR THR CYS GLY PRO PHE GLY ASN TRP
 TCT GAA GGA ACA AAC TTA ACT GGG ATT GAA GAA ACC ACC TGT GGA CCA TTT GGA AAC TGG

240
 250 SER PRO GLU PRO THR CYS GLN VAL ILE GLN CYS GLU PRO LEU SER ALA PRO ASP LEU
 TCA TCT CCA GAA CCA ACC TGT CAA GTG ATT CAG TGT GAG CCT CTA TCA GCA CCA GAT TTG

260
 270 GLY ILE MET ASN CYS SER HIS PRO LEU ALA SER PHE THR SER ALA CYS THR PHE
 GGG ATC ATG AAC TGT AGC CAT CCC CTG GCC AGC TTC ACC TTT ACC TCT GCA TGT ACC TTC

280
 290 ILE CYS SER GLU GLY THR GLU LEU ILE GLY LYS LYS THR ILE CYS GLU SER SER GLY
 ATC TGC TCA GAA GGA ACT GAG TTA ATT GGG AAG AAG AAA ACC ATT TGT GAA TCA TCT GGA

300
 310 ILE TRP SER ASN PRO SER PRO ILE CYS GLN LYS LEU ASP LYS SER PHE SER MET ILE LYS
 ATC TGG TCA AAT CCT AGT CCA ATA TGT CAA AAA TTG GAC AAA AGT TTC TCA ATG ATT AAG

320
 330 GLU GLY ASP TYR ASN PRO LEU PHE ILE PRO VAL ALA VAL MET VAL THR ALA PHE SER GLY
 GAG GGT GAT TAT AAC CCC CTC TTC ATT CCA GTG GCA GTC ATG GTT ACT GCA TTC TCT GGG

340 STOP TRANSFER SEQUENCE

.....

FIG. 2-1

GAATTCTCGAGCTCGTCCGACCGCCCTCTTGTGCAAGAACTCTGAGCCCCAGGTGCAGGAGGCTGAGGCCTGCAGAG

10
1 MET VAL PHE PRO TRP ARG CYS GLU GLY THR TYR TRP GLY
AGACTTGCAGAGAGACCCAGCAAGCC ATG GTG TTT CCA TGG AGA TGT GAG GGT ACT TAC TGG GGC

20 30 SIGNAL SEQUENCE
LEU TRP VAL TRP THR LEU LEU CYS CYS ASP PHE LEU ILE HIS
CTG TGG GTC TGG ACA CTG CTC TGT TGT GAC TTC CTG ATA CAC

40
N-TERMINUS
HIS GLY THR HIS CYS TRP THR TYR HIS TYR SER GLU LYS PRO MET ASN TRP GLU ASN ALA
CAT GGA ACT CAC TGT TGG ACT TAC CAT TAT TCT GAA AAG CCC ATG AAC TGG GAA AAT GCT

60 70
ARG LYS PHE CYS LYS GLN ASN TYR THR ASP LEU VAL ALA ILE GLN ASN LYS ARG GLU ILE
AGA AAG TTC TGC AAG CAA AAT TAC ACA GAT TTA GTC GCC ATA CAA AAC AAG AGA GAA ATT

80 90
GLU TYR LEU GLU ASN THR LEU PRO LYS SER PRO TYR TYR TRP ILE GLY ILE ARG LYS
GAG TAT TTA GAG AAT ACA TTG CCC AAA AGC CCT TAT TAC TAC TGG ATA GGA ATC AGG AAA

100 110
ILE GLY LYS MET TRP THR VAL GLY THR ASN LYS THR LEU THR LYS GLU ALA GLU ASN
ATT GGG AAA ATG TGG ACA TGG GTG GGA ACC AAC AAA ACT CTC ACT AAA GAA GCA GAG AAC

120 130
TRP GLY ALA GLY GLU PRO ASN ASN LYS SER LYS GLU ASP CYS VAL GLU ILE TYR ILE
TGG GGT GCT GGG GAG CCC AAC AAC AAG AAG TCC AAC GAG GAC TGT GTG GAG ATC TAT ATC

140 150
LYS ARG GLU ARG ASP SER GLY LYS TRP ASN ASP ALA CYS HIS LYS ARG LYS ALA ALA
AAG AGG GAA CGA GAC TCT TGT GGG AAA TGG AAC GAT GAC GCC TGT CAC AAA CGA AAG GCA GCT

FIG. 2--2

160 LEU [CYS] TYR THR ALA SER [CYS] GLN PRO GLY SER CYS ASN GLY ARG GLY GLU [CYS] VAL GLU
 CTC [TGC] TAC ACA GCC TCT [TGC] CAG CCA GGG TCT TGC AAT GGC CGT GGA GAA [TGT] GTG GAA
 170
 180 THR ILE ASN [ASN HIS THR] [CYS] ILE [CYS] ASP ALA GLY TYR TYR GLY PRO GLN [CYS] GLN TYR
 ACT ATC AAC AAT CAC ACG [TGC] ATC [TGT] GAT GCA GGG TAT TAC GGG CCC CAG [TGT] CAG TAT
 190
 200 VAL VAL GLN [CYS] GLU PRO LEU GLU ALA PRO GLU LEU GLY THR MET ASP [CYS] ILE HIS PRO
 GTG GTC CAG [TGT] GAG CCT TTG GAG GCC CCT GAG TTG GGT ACC ATG GAC [TGC] ATC CAC CCC
 210
 220 LEU GLY [ASN PHE SER] PHE GLN SER LYS [CYS] ALA PHE [ASN CYS SER] GLU GLY ARG GLU LEU
 TTG GGA AAC TTC AGC [TTC CAG TCC AAG] [TGT] GCT TTC AAC TGT TCT GAG GGA AGA GAG CTA
 230
 240 LEU GLY THR ALA GLU THR GLN [CYS] GLY ALA SER GLY [ASN TRP SER] SER PRO GLU PRO ILE
 CTT GGG ACT GCA GAA ACA CAG [TGT] GGA GCA TCT GGA AAC TGG TCA TCT CCA GAG CCA ATC
 250
 260 [CYS] GLN VAL VAL GLN [CYS] GLU PRO LEU GLU ALA PRO GLU LEU GLY THR MET ASP [CYS] ILE
 [TGC] CAA GTG GTC CAG [TGT] GAG CCT TTG GAG GCC CCT GAG TTG GGT ACC ATG GAC [TGC] ATC
 270
 280 HIS PRO LEU GLY [ASN PHE SER] PHE GLN SER LYS [CYS] ALA PHE [ASN CYS SER] GLU GLY ARG
 CAC CCC TTG GGA AAC TTC AGC [TTC CAG TCC AAG] [TGT] GCT TTC AAC TGT TCT GAG GGA AGA
 290
 300 GLU LEU LEU GLY THR ALA GLU THR GLN [CYS] GLY ALA SER GLY [ASN TRP SER] SER PRO GLU
 GAG CTA CTT GGG ACT GCA GAA ACA CAG [TGT] GGA GCA TCT GGA AAC TGG TCA TCT CCA GAG
 310
 320 PRO ILE [CYS] GLN GLU THR [ASN ARG SER] PHE SER LYS ILE LYS GLU GLY ASP TYR ASN [PRO]
 CCA ATC [TGC] CAA GAG ACA AAC AGA AGT [TTC TCA AAG ATC AAA GAA GGT GAC TAC AAC] CCC
 330

FIG. 2-3

340 STOP TRANSFER SEQUENCE 350
LEU PHE ILE PRO VAL ALA VAL MET VAL THR ALA PHE SER GLY LEU ALA PHE LEU ILE TRP
CTC TTC ATT CCT GTA GCC GTC ATG GTC ACC GCA TTC TCG GGG CTG GCA TTT CTC ATT TGG

360 370 372
LEU ALA ARG ARG LEU LYS LYS GLY LYS LYS SER GLN GLU ARG MET ASP PRO TYR OP
CTG GCA AGG CGG TTA AAA AAA GGC AAG AAA TCT CAA GAA AGG ATG GAT CCA TAC TGA

TTCATCCTTTGTGAAAGGAAAGCCCATGAAGTGCTAAAGACAAAACATTTGGAAAATAACGTCAAGTCCTCCCGTGAAGA
TTTTACAGCAGGCATCTCCACATTAGAGATGCAGTGTGTTGCTCAACGAATCTGGAAGGATTTCTTCATGACCAACA
GCTCCTCCTAATTTCCCTCGCTCATTCATCCCATTAACCCCTATCCCATATGTGTCTATACAGAGTAGTATTTTA
TCATCTTTTCTGTGGAGGAACAAGCAAAAGTGTTACTGTAGATAATAAAGACAGCTGCTTTTACTCTTTCTTAACTCT
TGTTTCCTAGTTCAATTCAGCACAGAAGCTAATGCCAAACACACAGTGAAAATATGATCCATGAGTAATTGGAAACTCAG
ACTCCTTCGGCATAGTACGTACCCCTATGTAACATCGACAAAAATCTTTCATTTCCACCTCCAAAGAACAGTGCTCTAT
TCAAGTTGGGAAAGTCCTACTTCTCTGTAGACCCCACTATCTGTGAGTGACAGCCACTGTAGCTGTTACATTAACCT
TCCCCATCTCCTTTTCCCTAGGAGAAATAATTCCACACACTGCACCCCATGATGGCCACCAACATCAAGAAGGGAAAA
TCTCCTGCATTGAGTTTGTAGTTTTTCCCTTCTTTATTAGATCTCTGATGGTTCCTTGAAGTCAGTGTCTCT
GATGATTATTAAATAGTTAATGATAACACAAACCACTCTCTTGGAGCTGATGTTATGAAGACAAACAGGTAGAAAAATTC
CTGGGCTCAGGCTGGAGTGACACCCCTTTTCTTTCCCTAACATCTTCTACTCAGATACCTAAATTTAAGATTCAGGACA
GCTGTCCCAACTCTTACCATGTCTTTTATAAAGTCTCTTAACTTGCTCCTTAACTTGCCCAACCTGAGGCTATCTCATTTTCTCGC
TTCACCTCTGCAAGGTTTATAACATGATGAATTTAAATACAAAAA

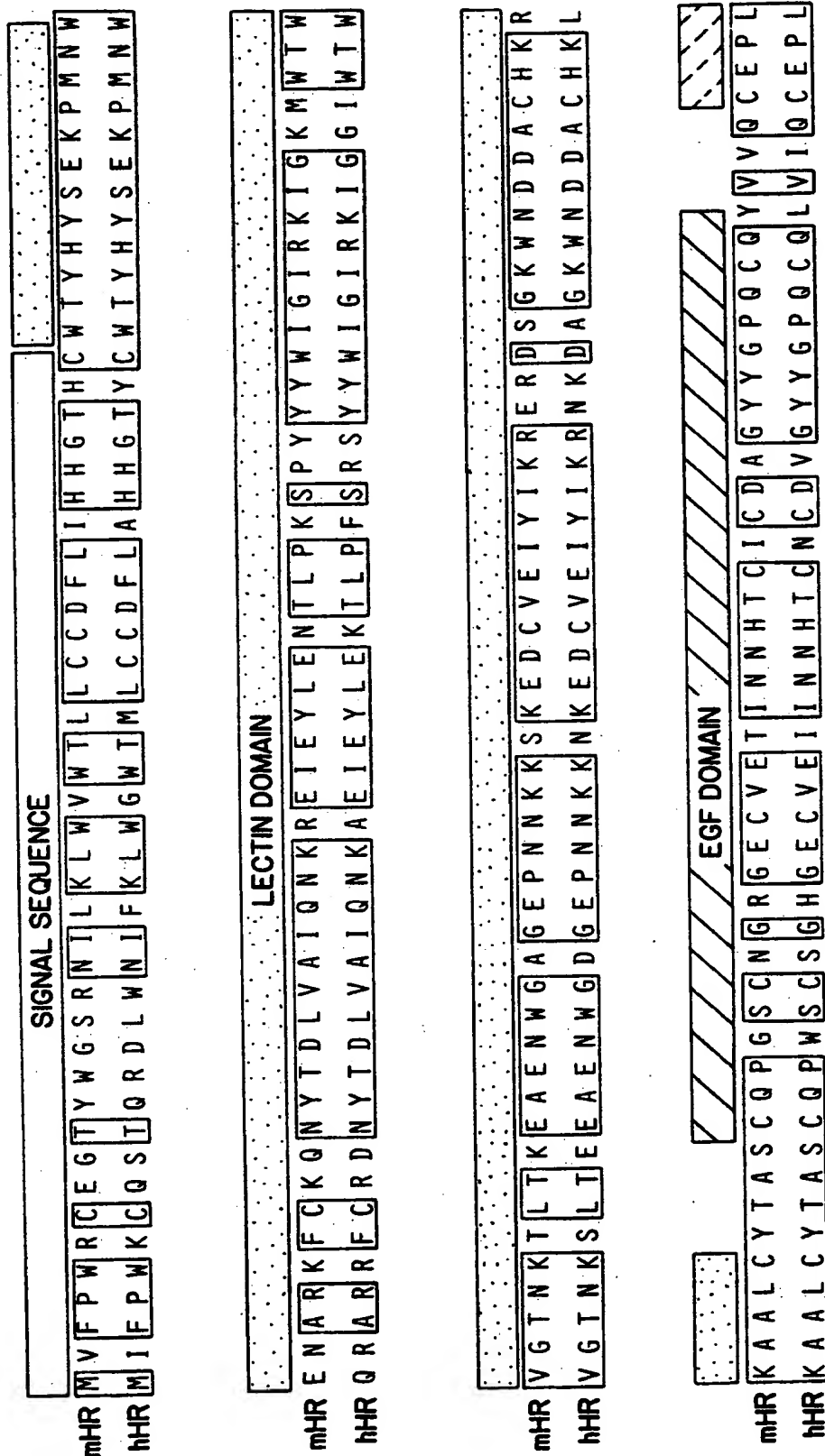


FIG.3-1

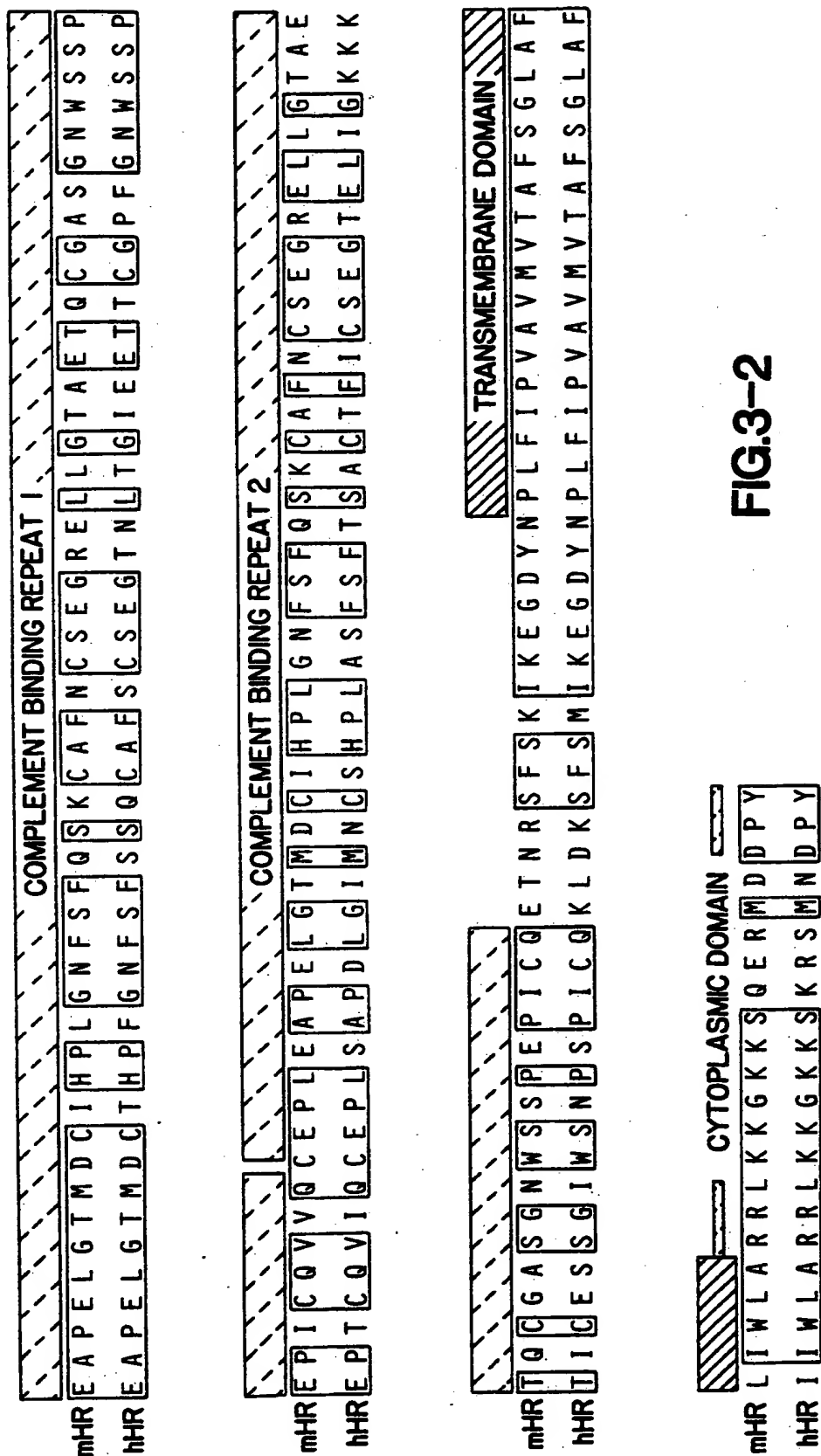


FIG.3-2

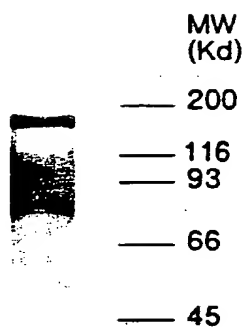


FIG.4A

T H K M K F K V V I L K
1 10 20 30
X T Y H Y S E K P M N W E N A R K F X K Q N Y T D L V A I Q N K X X I E Y L

FIG.4B

A A C A C
5' GAG AAG CCC ATG AAT TGG GAG AAT GC 3'

FIG.4C

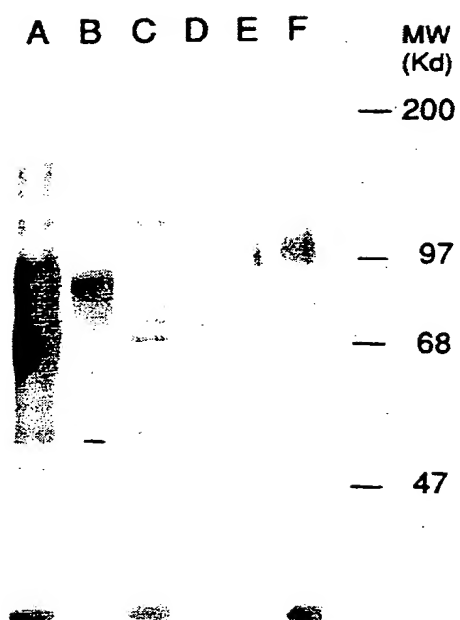


FIG. 5

FIG. 6A-1

DRICKAMER	G	39	181	1	121	61	121	121	121	1	0	0	0	0
MLHR														
HU. HEPLEC														
BARN. LEC														
RA. HEPLEC														
CH. HEPLEC														
HU. IGEREC														
RAHEPLEC2														
RA. ASGREC														
RA. IRP														
RA. MBP														
RA. MBDA														
RA. KCBP														
FLYLEC														
RAB. SURF														

DRICKAMER	G	84	224	45	164	104	166	164	164	47	165	44	43	48	45
MLHR															
HU. HEPLEC															
BARN. LEC															
RA. HEPLEC															
CH. HEPLEC															
HU. IGEREC															
RAHEPLEC2															
RA. ASGREC															
RA. IRP															
RA. MBP															
RA. MBDA															
RA. KCBP															
FLYLEC															
RAB. SURF															

[illegible]

FIG. 6A-2

MLHR	160
SNOTCH	1021
S. PURP.	61
PRO. Z	1
FACT. X	61
FACT. VII	61
FACT. IX	1
LIN-12	361
FACT. XII	121
MU. EGF	841

FIG. 6B

[illegible]

FIG. 6C-2

PROTEIN MOTIFS IN THE LYMPHOCYTE HOMING RECEPTOR

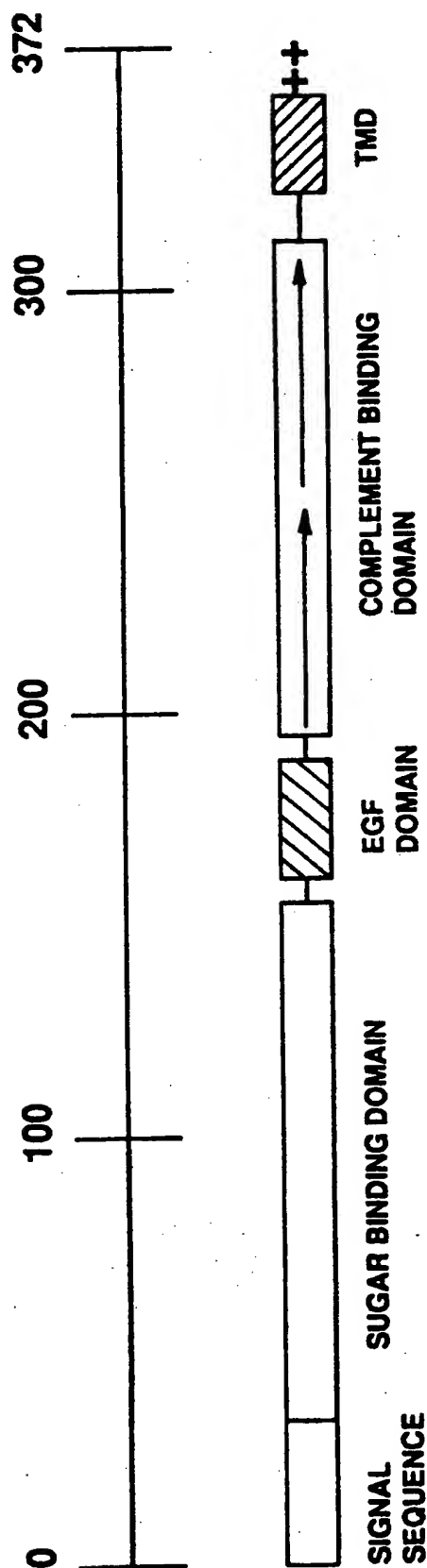


FIG.7

Murine PLN Homing Receptor IgG Chimeras

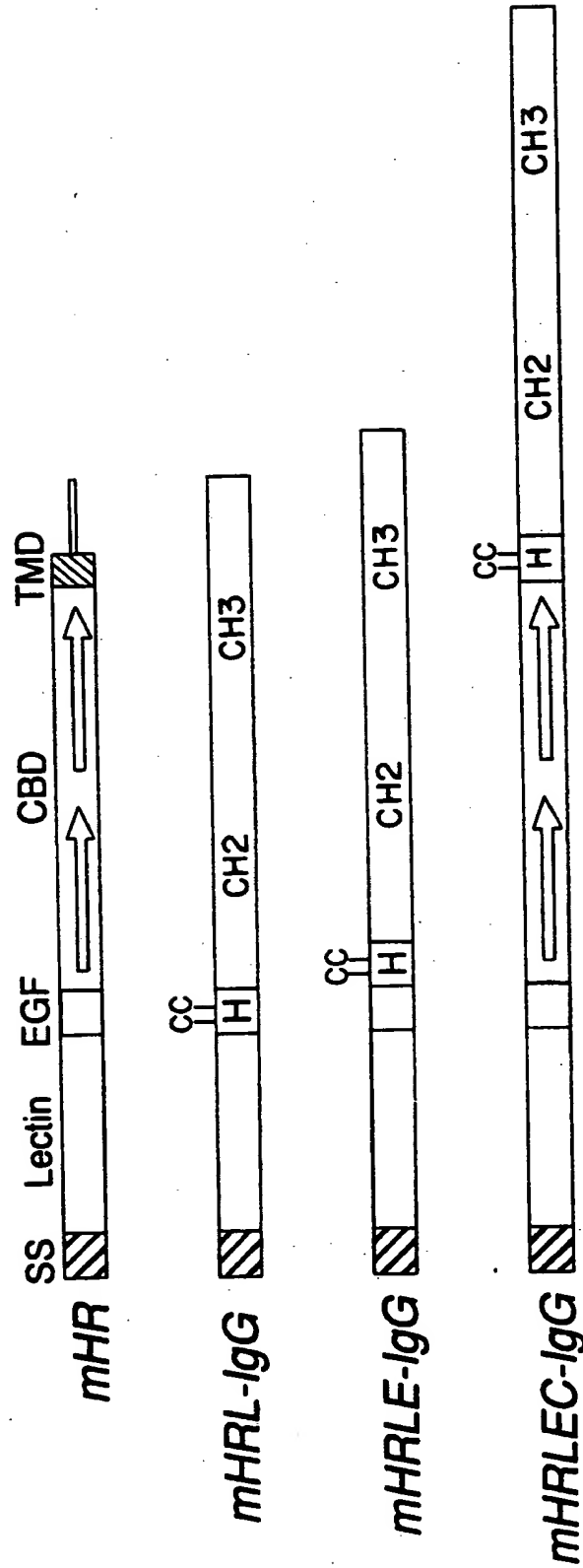
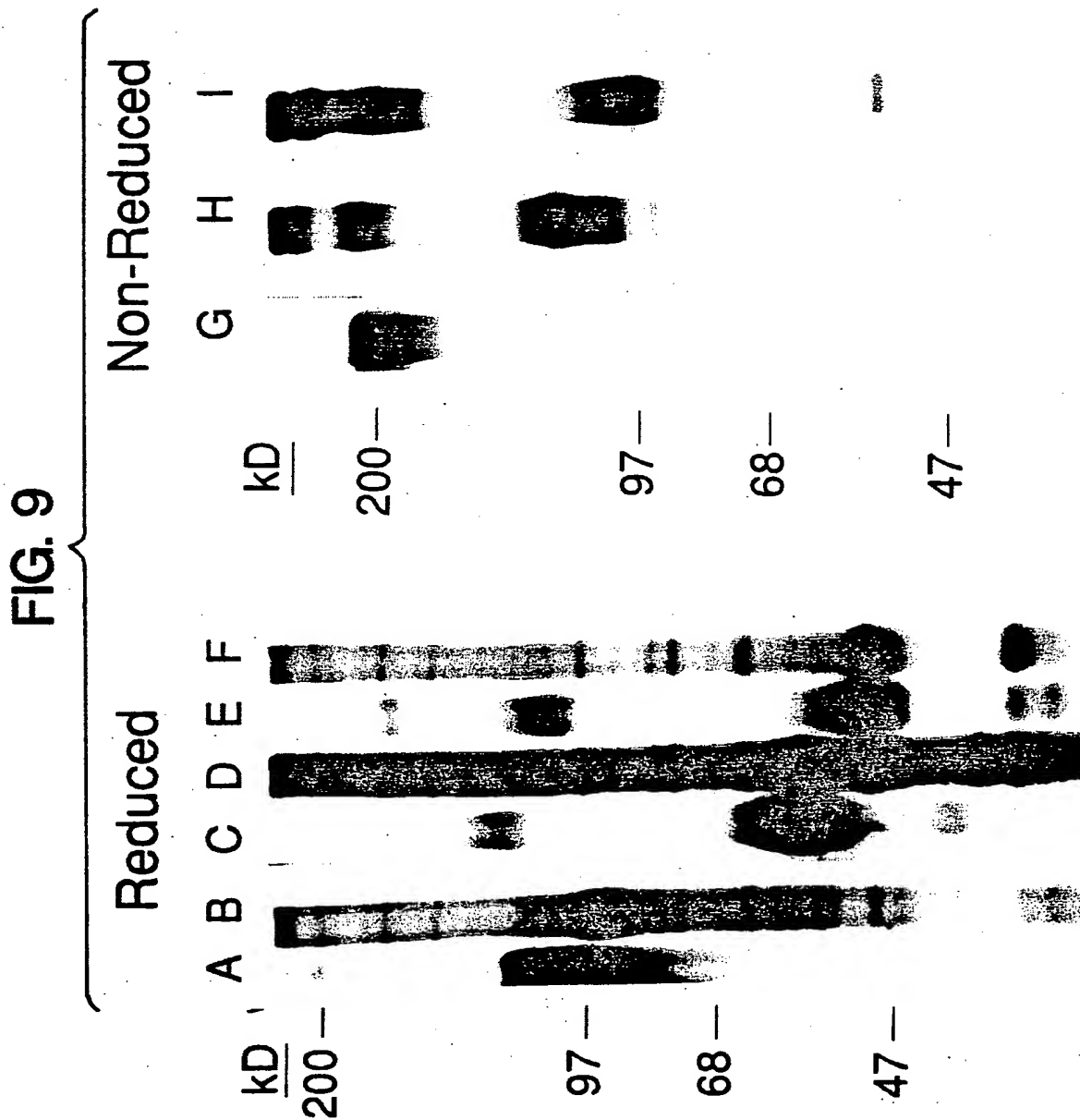
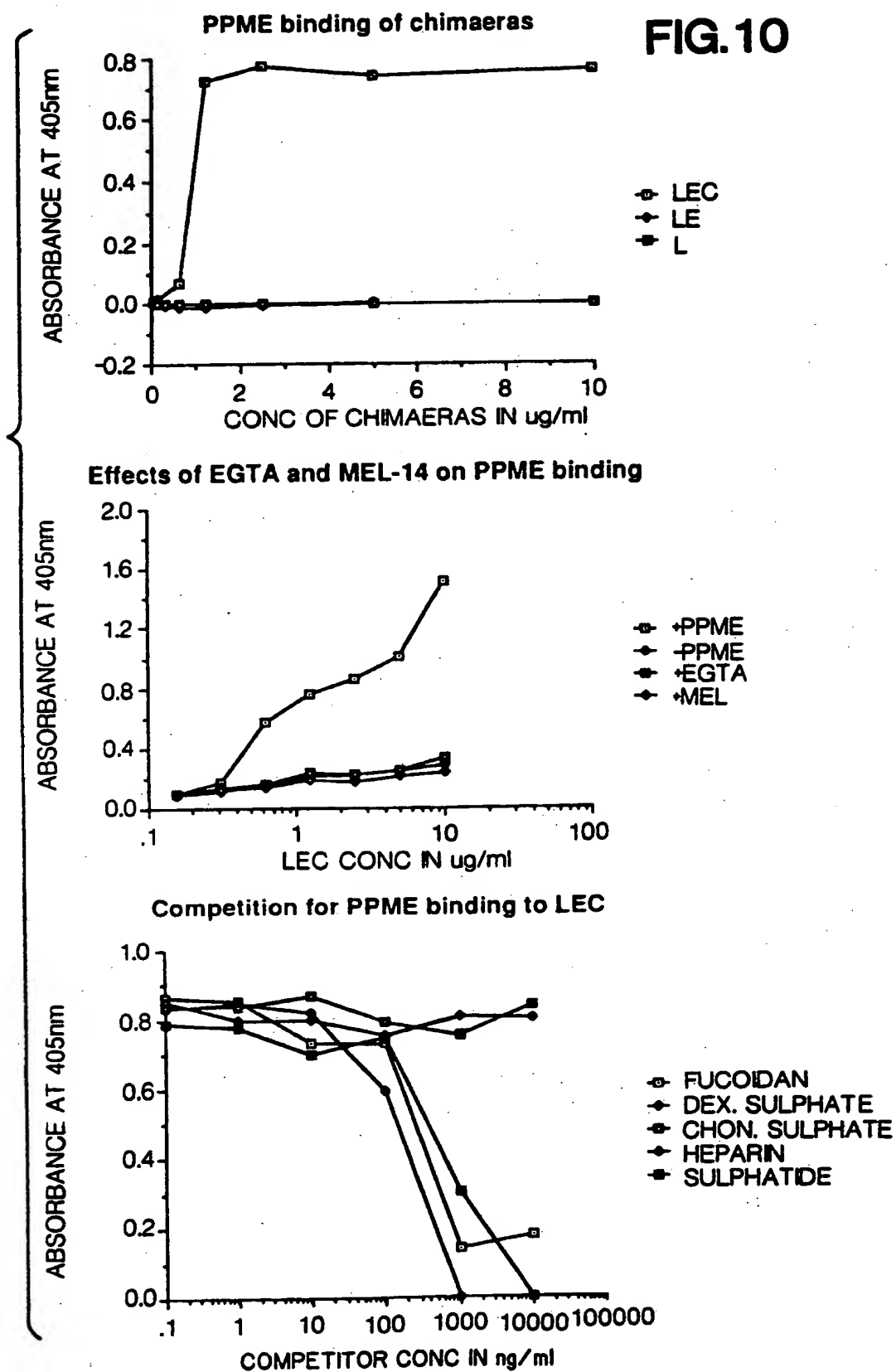


FIG.8





HYBRID IMMUNOGLOBULINS

This is a continuation-in-part of U.S. Ser. No. 07/315,015, filed Feb. 23, 1989.

BACKGROUND OF THE INVENTION

This invention relates to novel ligand binding molecules and receptors, and to compositions and methods for improving the circulating plasma half-life of ligand binding molecules. In particular, this invention also relates to hybrid immunoglobulin molecules, to methods for making and using these immunoglobulins, and to nucleic acids encoding them.

Immunoglobulins are molecules containing polypeptide chains held together by disulfide bonds, typically having two light chains and two heavy chains. In each chain, one domain (V) has a variable amino acid sequence depending on the antibody specificity of the molecule. The other domains (C) have a rather constant sequence common among molecules of the same class. The domains are numbered in sequence from the amino-terminal end.

The immunoglobulin gene superfamily consists of molecules with immunoglobulin-like domains. Members of this family include class I and class II major histocompatibility antigens, immunoglobulins, T-cell receptor α , β , γ and δ chains, CD1, CD2, CD4, CD8, CD28, the γ , δ and ϵ chains of CD3, OX-2, Thy-1, the intercellular or neural cell adhesion molecules (I-CAM or N-CAM), lymphocyte function associated antigen-3 (LFA-3), neurocytoplasmic protein (NCP-3), poly-Ig receptor, myelin-associated glycoprotein (MAG), high affinity IgE receptor, the major glycoprotein of peripheral myelin (Po), platelet derived growth factor receptor, colony stimulating factor-1 receptor, macrophage Fc receptor, Fc gamma receptors and carcinoembryonic antigen.

It is known that one can substitute variable domains (including hypervariable regions) of one immunoglobulin for another, and from one species to another. See, for example, EP 0 173 494; EP 0 125 023; Munro, *Nature* 312 (13 Dec. 1984); Neuberger et al., *Nature* 312 (13 Dec. 1984); Sharon et al., *Nature* 309 (May 24, 1984); Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Morrison et al., *Science* 229:1202-1207 (1985); and Boulianne et al., *Nature* 312:643-646 (Dec. 13, 1984).

Morrison et al., *Science* 229:1202-1207 (1985) teaches the preparation of an immunoglobulin chimera having a variable region from one species fused to an immunoglobulin constant region from another species. This reference suggests molecules having immunoglobulin sequences fused with non-immunoglobulin sequences (for example enzyme sequences), however the reference teaches only immunoglobulin variable domains attached to the non-immunoglobulin sequence. Morrison et al., EP 0 173 494 teaches a similar chimera. While the term "receptor" is used by the authors, and the background section refers to "receptors such as immunoglobulins, enzymes and membrane proteins", the stated "receptors of interest" include "B-cell and T-cell receptors, more particularly, immunoglobulins, such as IgM, IgG, IgA, IgD and IgE, as well as the various subtypes of the individual groups" (page 3 lines 10-13). The disclosure of this reference is specific to immunoglobulin chimeras (see for example page 3, lines 21-30).

It has also been shown that it is possible to substitute immunoglobulin variable-like domains from two members of the immunoglobulin gene superfamily—CD4 and the T cell receptor—for a variable domain in an immunoglobulin; see e.g. Capon et al., *Nature* 337:525-531, 1989; Traunecker et al., *Nature* 339:68-70, 1989; Gascoigne et al., *Proc. Nat. Acad. Sci.* 84:2936-2940, 1987, and published European application EPO 0 325 224 A2.

A large number of proteinaceous substances are known to function by binding specifically to target molecules. These target molecules are generally, but need not be, proteins. The substances which bind to target molecules or ligands are referred to herein as ligand binding partners, and include receptors and carrier proteins, as well as hormones cellular adhesive proteins, tissue-specific adhesion factors, lectin binding molecules growth factors, enzymes, nutrient substances and the like.

Lymphocytes are examples of cells which are targeted to specific tissues. Lymphocytes are mediators of normal tissue inflammation as well as pathologic tissue damage such as occurs in rheumatoid arthritis and other autoimmune diseases. Vertebrates have evolved a mechanism for distributing lymphocytes with diverse antigenic specificities to spatially distinct regions of the organism (Butcher, E. C., *Curr. Top. Micro. Immunol.* 128, 85 (1986); Gallatin, W. M., et al., *Cell* 44, 673 (1986); Woodruff, J. J., et al., *Ann. Rev. Immunol.* 5, 201 (1987); Duijvestijn, A., et al., *Immunol. Today* 10, 23 (1989); Yednock, T. A., et al., *Adv. Immunol.* 44: 313-78 (in press) (1989)).

This mechanism involves the continuous recirculation of the lymphocytes between the blood and the lymphoid organs. The migration of lymphocytes between the blood, where the cells have the greatest degree of mobility, and the lymphoid organs, where the lymphocytes encounter sequestered and processed antigen, is initiated by an adhesive interaction between receptors on the surface of the lymphocytes and ligands on the endothelial cells of specialized postcapillary venules, e.g., high endothelial venules (HEV) and the HEV-like vessels induced in chronically inflamed synovium.

The lymphocyte adhesion molecules have been specifically termed homing receptors, since they allow these cells to localize in or "home" to particular secondary lymphoid organs.

Candidates for the lymphocyte homing receptor have been identified in mouse, rat and human (Gallatin, W. M., et al., *Nature* 303, 30 (1983); Rasmussen, R. A., et al., *J. Immunol.* 135, 19 (1985); Chin, Y. H., et al., *J. Immunol.* 136, 2556 (1986); Jalkanen, S., et al., *Eur. J. Immunol.* 10, 1195 (1986)). The following literature describes work which has been done in this area through the use of a monoclonal antibody, termed Mel 14, directed against a purported murine form of a lymphocyte surface protein (Gallatin, W. M., et al., supra; Mountz, J. D., et al., *J. Immunol.* 140, 2943 (1988); Lewinsohn, D. M., et al., *J. Immunol.* 138, 4313 (1987); Siegelman, M., et al., *Science* 231, 823 (1986); St. John, T., et al., *Science* 231, 845 (1986)).

Immunoprecipitation experiments have shown that this antibody recognizes a diffuse, ~90,000 dalton cell surface protein on lymphocytes (Gallatin, W. M., et al., supra) and a ~100,000 dalton protein on neutrophils (Lewinsohn, D. M., et al., supra).

A partial sequence—13 residues—for a purported lymphocyte homing receptor identified by radioactively labeled amino acid sequencing of a Mel.14 antibody-defined glycoprotein was disclosed by Siegelman et al. (Siegelman, M., et al., *Science* 231, 823 (1986)).

Lectins are proteins with a carbohydrate-binding domain found in a variety of animals, including humans as well as the acorn barnacle and the flesh fly. The concept of lectins functioning in cell adhesion is exemplified by the interaction of certain viruses and bacteria with eucaryotic host cells (Paulson, J. C., *The Receptors* Vol. 2 P. M. Conn, Eds. (Academic Press, NY, 1985), pp. 131; Sharon, N., *FEBS Lett.* 217, 145 (1987)). In eucaryotic cell-cell interactions, adhesive functions have been inferred for endogenous lectins in a variety of systems (Grabel, L., et al., *Cell* 17, 477 (1979); Fenderson, B., et al., *J. Exp. Med.* 160, 1591 (1984); Kunemund, V., *J. Cell Biol.* 106, 213 (1988); Bischoff, R., *J. Cell Biol.* 102, 2273 (1986); Crocker, P. R., et al., *J. Exp. Med.* 164, 1862 (1986); including invertebrate (Glabe, C. G., et al., *J. Cell Biol.* 94, 123 (1982); DeAngelis, P., et al., *J. Biol. Chem.* 262, 13946 (1987)) and vertebrate fertilization (Bleil, J. D., et al., *Proc. Natl. Acad. Sci., U.S.A.* 85, 6778 (1988); Lopez, L. C., et al., *J. Cell Biol.* 101, 1501 (1985)). The use of protein-sugar interactions as a means of achieving specific cell recognition appears to be well known.

The literature suggests that a lectin may be involved in the adhesive interaction between the lymphocytes and their ligands (Rosen, S. D., et al., *Science* 228, 1005 (1985); Rosen, S. D., et al., *J. Immunol.* 142 (6):1895-1902 (1989); Stoolman, L. M., et al., *J. Cell Biol.* 96, 722 (1983); Stoolman, L. M., et al., *J. Cell Biol.* 99, 1535 (1984); Yednock, T. A., et al., *J. Cell Biol.* 104, 725 (1987); Stoolman, L. M., et al., *Blood* 70, 1842 (1987); A related approach by Brandley, B. K., et al., *J. Cell Biol.* 105, 991 (1987); and Yednock, T. A., et al., *J. Cell Biol.* 104, 725 (1987)).

The character of a surface glycoprotein that may be involved in human lymphocyte homing was investigated with a series of monoclonal and polyclonal antibodies generically termed Hermes. These antibodies recognized a ~90,000 dalton surface glycoprotein that was found on a large number of both immune and non-immune cell types and which, by antibody pre-clearing experiments, appeared to be related to the Mel 14 antigen. (Jalkanen, S., et al., *Ann. Rev. Med.* 38, 467-476 (1987); Jalkanen, S., et al., *Blood* 66(3), 577-582 (1985); Jalkanen, S., et al., *J. Cell Biol.* 105, 983-990 (1987); Jalkanen, S., et al., *Eur. J. Immunol.* 16, 1195-1202 (1986)).

Epidermal growth factor-like domains have been found on a wide range of proteins, including growth factors, cell surface receptors, developmental gene products, extracellular matrix proteins, blood clotting factors, plasminogen activators, and complement (Doolittle, R. F., et al., *CSH Symp.* 51, 447 (1986)).

A lymphocyte cell surface glycoprotein (referred to hereafter as the "LHR") has been characterized which mediates the binding of lymphocytes to the endothelium of lymphoid tissue. Full length cDNA clones and DNA encoding the human and the murine LHR (HuLHR and MLHR, respectively) have been identified and isolated, and moreover this DNA is readily expressed by recombinant host cells. The nucleotide and amino acid sequence of the human LHR (HuLHR) is shown in FIG. 1. The nucleotide and amino acid sequence of the murine LHR (MLHR) is shown in FIG.

2. Also provided are LHR having variant amino acid sequences or glycosylation not otherwise found in nature, as well as other derivatives of the LHR having improved properties including enhanced specific activity and modified plasma half-life, as well as enabling methods for the preparation of such variants.

It is shown herein that the LHR is a glycoprotein which contains the following protein domains: a signal sequence, a carbohydrate binding domain, an epidermal growth factor-like (egf) domain, at least one and preferably two complement binding domain repeat, a transmembrane binding domain (TMD), and a charged intracellular or cytoplasmic domain. The LHR of this invention contains at least one but not necessarily all of these domains.

A successful strategy in the development of drugs for the treatment of many abnormalities in ligand-binding partner interactions has been the identification of antagonists which block the binding or interaction between ligand and binding partner. One approach has been to use an exogenous binding partner as a competitive antagonist for the native binding partner. However, many ligand binding partners are cell membrane proteins which are anchored in the lipid bilayer of cells. The presence of membrane components is typically undesirable from the standpoint of manufacturing and purification. In addition, since these molecules are normally present only on cell surfaces, it would be desirable to produce them in a form which is more stable in the circulation. Additionally, even truncated or soluble ligand binding partners may not be optimally effective as therapeutics since they possess a relatively short in vivo plasma half-life, may not cross the placental or other biological barriers, and since merely sequestering their ligand recognition site without delivering an effector function may be inadequate for therapeutic purposes.

Accordingly, it is an object of this invention to produce ligand binding partners fused to moieties which serve to prolong the in vivo plasma half-life of the ligand binding partner, such as immunoglobulin domains or plasma proteins, and facilitate its purification by protein A. It is a further object to provide novel hybrid immunoglobulin molecules which combine the adhesive and targeting characteristics of a ligand binding partner with immunoglobulin effector functions such as complement binding, cell receptor binding and the like. Yet another object is to provide molecules with novel functionalities such as those described above for therapeutic use, or for use as diagnostic reagents for the in vitro assay of the ligand binding partners or their targets. It is another object to provide multifunctional molecules in which a plurality of ligand binding partners (each of which may be the same or different) are assembled, whereby the molecules become capable of binding and/or activating more than one ligand.

In particular, it is an objective to prepare molecules for directing ligand binding partners such as toxins, cell surface partners, enzymes, nutrient substances, growth factors, hormones or effector molecules such as the constant domain-like portions of a member of the immunoglobulin gene superfamily to cells bearing ligands for the ligand binding partners, and for use in facilitating purification of the ligand binding partners.

Another object of this invention is to provide ligand binding partner-immunoglobulin hybrid heteropolymers, especially heterodimers and heterotetramers, which are used in the targeting of therapeutic moieties to spe-

cific tissues and ligands. For example, a hybrid immunoglobulin consisting of one LHR-IgG chain and one CD4-IgG chain can be used to target CD4-IgG to tissues infected by viruses such as the human immunodeficiency virus (HIV). Similarly, a molecule having a ligand binding partner, plasma protein portion combined with a toxin-plasma protein portion is used to deliver the toxin to desired tissues.

It is another object to provide a method for expression of these molecules in recombinant cell culture.

SUMMARY OF THE INVENTION

The objects of this invention are accomplished by providing novel polypeptides comprising a ligand binding partner fused to a stable plasma protein which is capable of extending the in vivo plasma half-life of the ligand binding partner when present as a fusion with the ligand binding partner, in particular wherein such a stable plasma protein is an immunoglobulin constant domain. DNA encoding the polypeptides, cultures and methods for making the polypeptides are also provided.

In most cases where the stable plasma protein is normally found in a multimeric form, e.g., immunoglobulins or lipoproteins, in which the same or different polypeptide chains are normally disulfide and/or noncovalently bound to form an assembled multichain polypeptide, the fusions herein containing the ligand binding partner also will be produced and employed as a multimer having substantially the same structure as the stable plasma protein precursor. These multimers will be homogeneous with respect to the ligand binding partner they comprise, or they may contain more than one ligand binding partner. Furthermore, they may contain one or more ligand binding partner moieties.

In a preferred embodiment in which the stable plasma protein is an immunoglobulin chain, the ligand binding partner will be substituted into at least one chain, and ordinarily for the variable region of the immunoglobulin or suitable fragment thereof. However, it will be understood that this invention also comprises those fusions where the same or different ligand binding partners are substituted into more than one chain of the immunoglobulin. If the ligand binding partners are different, then the final assembled multichain polypeptide is capable of crosslinking ligands in a fashion that may not be possible with multifunctional antibodies having native variable regions.

A particular multichain fusion of this sort is one in which the variable region of one immunoglobulin chain has been substituted by the ligand binding region of a first receptor such as CD4 while the variable region of another immunoglobulin chain has been substituted by a binding functionality of the LHR, both immunoglobulin chains being associated with one another in substantially normal fashion.

The fusions of this invention may be further modified by linking them through peptidyl or in vitro generated bonds to an additional therapeutic moiety such as a polypeptide toxin, a diagnostic label or other functionality.

The fusions of this invention are made by transforming host cells with nucleic acid encoding the fusion, culturing the host cell and recovering the fusion from the culture. Also provided are vectors and nucleic acid encoding the fusion, as well as therapeutic and diagnostic compositions comprising them.

In certain respects this invention is directed to LHR per se. The LHR of this invention is full-length, mature

LHR, having the amino acid sequence described herein at FIGS. 1 and 2, and naturally occurring alleles, covalent derivatives made by in vitro derivatization, or predetermined amino acid sequence or glycosylation variants thereof.

The novel compositions provided herein are purified and formulated in pharmacologically acceptable vehicles for administration to patients in need of antiviral, neuromodulatory or immunomodulatory therapy, and for use in the modulation of cell adhesion. This invention is particularly useful for the treatment of patients having receptor-mediated abnormalities. In addition, the compositions provided herein are useful intermediates in the purification of the ligand binding partner from recombinant cell culture, wherein antibodies or other substances capable of binding the stable plasma protein component are used to absorb the fusion, or are useful in diagnostic assays for the ligand binding partner wherein the stable plasma protein serves as an indirect label.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 depicts the amino acid and DNA sequence of the Human LHR (HuLHR).

FIG. 2 depicts the amino acid and DNA sequence of the Murine LHR (MLHR).

FIG. 3 shows a comparison between the amino acid sequences for the mature HuLHR and MLHR.

FIGS. 4A-4C show the isolation and N-terminal sequencing of the MLHR. FIG. 4A shows an SDS-polyacrylamide gel of material purified from a detergent extract of murine spleens by Mel 14 monoclonal antibody affinity chromatography. FIG. 4B shows the results of the subjection of the 90,000 dalton band of FIG. 4A to gas phase Edman degradation. The residues underlined between amino acids 7 and 15 were chosen to produce the oligonucleotide probe shown in FIG. 4C. FIG. 4C shows a 32-fold redundant 26-mer oligonucleotide probe.

FIG. 5 shows the transient expression of the MLHR cDNA clone. Lanes A-F signify the following: A. Lysates of 293 cells transfected with a MLHR expression plasmid immunoprecipitated with Mel 14 monoclonal antibody. B. Supernatants of 293 cells transfected with a MLHR expression plasmid immunoprecipitated with Mel 14 monoclonal antibody. C. Lysates of 293 cells transfected with a plasmid expressing the HIV gp120 envelope glycoprotein immunoprecipitated with the Mel 14 monoclonal antibody. D. Supernatants of 293 cells transfected with the HIV envelope expression plasmid immunoprecipitated with the Mel 14 monoclonal antibody. E. Supernatants of 38C13 cells immunoprecipitated with the Mel 14 monoclonal antibody. F. Lysates of 38C13 cells surface labeled with I^{125} and immunoprecipitated with the Mel 14 monoclonal antibody.

FIGS. 6A-6C show protein sequences which are heterologous but functionally comparable to the MLHR. Those lines labelled "MLHR" correspond to the MLHR of FIG. 2. FIG. 6A compares carbohydrate-binding domains; FIG. 6B compares epidermal growth factor domains; and FIG. 6C compares complement binding factor domains.

FIG. 7 is a schematic of protein domains found in the LHR, including the signal sequence, carbohydrate binding domain, epidermal growth factor (egf) domain, two complement binding domain repeats (arrows), trans-

membrane binding domain (TMD), and charged intracellular domain.

FIG. 8 shows the construction of MLHR-IgG chimeras containing the lectin, lectin-egf, and lectin-egf-complement regulatory motifs.

FIG. 9 shows the gel electrophoresis of the products of the expression and purification of the MLHR-IgG chimeras.

FIG. 10 shows polyphosphomannan ester (PPME) binding analysis of various MLHR-IgG chimeras.

DETAILED DESCRIPTION

Ligand binding partners as defined herein are proteins known to function to bind specifically to target ligand molecules, and are generally found in their native state as secreted or membrane bound polypeptides; membrane-bound ligand binding partners typically include a hydrophobic transmembrane region or phospholipid anchor. Ligand binding partners include receptors and carrier proteins, as well as hormones, cellular adhesive proteins (proteins which direct or induce the adhesion of one cell to another), lectin binding molecules, growth factors, enzymes, nutrient substances and the like. CD antigens which are not members of the immunoglobulin gene superfamily or otherwise excluded as set forth above are suitable ligand binding partners. Knapp et al., *Immunology Today* 10 (8):253-258, 1989, specifically incorporated by reference. The platelet growth factor receptor and insulin receptor may optionally be ligand binding partners. Ligand binding partners include not only the full length native form, but truncations or other amino acid sequence variants that remain capable of binding to the normal ligand.

As used herein, the term "ligand binding partner" specifically excludes polymorphic and nonpolymorphic members of the immunoglobulin gene superfamily, and proteins which are homologous thereto, such as class I and class II major histocompatibility antigens, immunoglobulins, T-cell receptor α , β , γ and δ chains, CD1, CD2, CD4, CD8, CD28, the γ , δ and ϵ chains of CD3, OX-2, Thy-1, the intercellular or neural cell adhesion molecules (I-CAM or N-CAM), lymphocyte function associated antigen-3 (LFA-3), neurocytoplasmic protein (NCP-3) poly-Ig receptor myelin-associated glycoprotein (MAG), high affinity IgE receptor, the major glycoprotein of peripheral myelin (Po), platelet derived growth factor receptor, colony stimulating factor-1 receptor, macrophage Fc receptor, Fc gamma receptors and carcinoembryonic antigen. Homologous to a member of the immunoglobulin gene superfamily, for the purposes of this exclusion only, means having the sequence of a member of the immunoglobulin gene superfamily or having a sequence therewithin which has substantially the same (or a greater degree of) amino acid sequence homology to a known member of the superfamily as the specific examples given above have to the sequence of an immunoglobulin variable or constant domain. Note that this does not exclude embodiments in which a ligand binding partner fusion is assembled into a multimer with, in addition, a member or fusion of a member of the immunoglobulin gene superfamily.

Also specifically excluded from the term "ligand binding partner" are multiple subunit (chain) polypeptides encoded by discrete genes (genes which do not encode a single chain precursor polypeptide leading to the multiple subunit polypeptide), with at least one

subunit of the polypeptide being ordinarily inserted into the cell membrane, including cellular receptors (e.g., integrins) for extracellular matrix molecules, as exemplified in U.S. Ser. No. 07/290,224 filed Dec. 22, 1988. Note that this does not exclude embodiments in which a ligand binding partner fusion is assembled into a multimer with, in addition, a multiple subunit polypeptide or fusion of a multiple subunit polypeptide as defined in this paragraph.

Stable plasma proteins are proteins typically having about from 30 to 2,000 residues, which exhibit in their native environment an extended half-life in the circulation, i.e. greater than about 20 hours. Examples of suitable stable plasma proteins are immunoglobulins, albumin, lipoproteins, apolipoproteins and transferrin. The ligand binding partner typically is fused to the plasma protein at the N-terminus of the plasma protein or fragment thereof which is capable of conferring an extended half-life upon the ligand binding partner. The ligand binding partner generally is fused at its native C-terminus to the plasma protein. However, on occasion it may be advantageous to fuse a truncated form of the ligand binding partner (in which the transmembrane and cytoplasmic regions have been deleted) to a portion of the stable protein that exhibits a substantially hydrophobic hydropathy profile, typically the first site in the mature stable protein in which a hydrophobic region having greater than about 20 residues appears. Such sites are present in transferrin and are quite common in albumin and apolipoproteins and should present no difficulty in identification. As much of the remainder of the stable protein as is required to confer extended plasma half-life on the ligand binding partner is then incorporated into the fusion. Increases of greater than about 100% on the plasma half-life of the ligand binding partner are satisfactory.

In some preferred embodiments, the binding partner is an LHR. The LHR is defined as a polypeptide having a qualitative biological activity in common with the LHR of FIG. 1 or FIG. 2, and which preferably contains a domain greater than about 70% homologous with the carbohydrate binding domain, the epidermal growth factor domain, or the carbohydrate binding domain of the LHR of FIG. 1 or FIG. 2.

Homology with respect to a LHR is defined herein as the percentage of residues in the candidate sequence that are identical with the residues in the carbohydrate binding domain, the epidermal growth factor domain, or the complement binding domains in FIG. 1 or FIG. 2 after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology.

Included within the scope of the LHR as that term is used herein are LHRs having the amino acid sequences of the HuLHR or MLHR as set forth in FIG. 1 or 2, deglycosylated or unglycosylated derivatives of the LHR, homologous amino acid sequence variants of the sequence of FIG. 1 or 2, and homologous in vitro-generated variants and derivatives of the LHR, which are capable of exhibiting a biological activity in common with the LHR of FIG. 1 or FIG. 2.

LHR or LHR-fragment biological activity is defined as either 1) immunological cross-reactivity with at least one epitope of the LHR, or 2) the possession of at least one adhesive, regulatory or effector function qualitatively in common with the LHR.

One example of the qualitative biological activities of the LHR is its binding to ligands on the specialized high endothelial cells of the lymphoid tissues. Also, it fre-

quently requires a divalent cation such as calcium for ligand binding.

Immunologically cross-reactive as used herein means that the candidate polypeptide is capable of competitively inhibiting the qualitative biological activity of the LHR having this activity with polyclonal antisera raised against the known active analogue. Such antisera are prepared in conventional fashion by injecting goats or rabbits, for example, subcutaneously with the known active analogue in complete Freund's adjuvant, followed by booster intraperitoneal or subcutaneous injection in incomplete Freund's.

Structurally, as shown in FIG. 3, the LHR includes several domains which are identified as follows (within ± 10 residues): a signal sequence (residues 20-32), which is followed by a carbohydrate binding domain (identified in FIG. 3 as a "lectin" domain) (residues 39-155), an epidermal growth factor (egf) domain (residues 160-193), a complement factor binding domain (residues 197-317), a transmembrane binding domain (TMD) (residues 333-355), and a cytoplasmic domain (residues 356-372). The boundary for the LHR extracellular domain generally is at, or within about 30 residues of, the N-terminus of the transmembrane domain, and is readily identified from an inspection of the LHR sequence. Any or all of these domains are utilized in the practice of this invention.

FIGS. 6A-6C show a variety of proteins having some homology to three of these domains. FIG. 6A shows carbohydrate binding domains, FIG. 6B shows epidermal growth factor domains, and FIG. 6C shows somewhat homologous complement binding domains.

A comparison of the amino sequences of HuLHR and MLHR is presented in FIG. 3, and shows a high degree of overall sequence homology ($\sim 83\%$). The degrees of homology between the various domains found in the HuLHR versus the MLHR, however, are variable. For example, the degree of sequence conservation between the MLHR and the HuLHR in both the carbohydrate-binding and egf domains is approximately 83%, while the degree of conservation in the first complement binding repeat falls to 79% and only 63% in the second repeat, for an overall complement binding domain homology of $\sim 71\%$. Furthermore, while the two MLHR complement binding domain repeats are identical, those in the HuLHR have differences, and differ as well to the murine repeats. Interestingly, the degree of conservation between the two receptors in the transmembrane sequence and surrounding regions is virtually identical, with only one conservative hydrophobic substitution, probably within the transmembrane anchor region.

The surface glycoprotein discussed above that is recognized by the series of monoclonal and polyclonal antibodies generically termed Hermes is specifically excluded from the scope of this invention.

Immunoglobulins and certain variants thereof are known and many have been prepared in recombinant cell culture. For example, see U.S. Pat. No. 4,745,055; EP 256,654; Faulkner et al., *Nature* 298:286 (1982); EP 120,694; EP 125,023; Morrison, J. Immun. 123:793 (1979); Köhler et al., P.N.A.S. USA 77:2197 (1980); Raso et al., *Cancer Res.* 41:2073 (1981); Morrison et al., *Ann Rev. Immunol.* 2:239 (1984); Morrison, *Science* 229:1202 (1985); Morrison et al., P.N.A.S. USA 81:6851 (1984); EP 255,694; EP 266,663; and WO 88/03559. Reassorted immunoglobulin chains also are known. See for example U.S. Pat. No. 4,444,878; WO 88/03565; and EP 68,763 and references cited therein.

Ordinarily, the ligand binding partner is fused C-terminally to the N-terminus of the constant region of immunoglobulins in place of the variable region(s) thereof, however N-terminal fusions of the binding partner are also desirable. The transmembrane regions or lipid or phospholipid anchor recognition sequences of ligand binding partners comprising such regions or sequences are preferably inactivated or deleted prior to fusion.

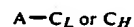
Typically, such fusions retain at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain. This ordinarily is accomplished by constructing the appropriate DNA sequence and expressing it in recombinant cell culture. Alternatively, however the polypeptides of this invention may be synthesized according to known methods.

The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion or binding characteristics of the binding partner. The optimal site will be determined by routine experimentation.

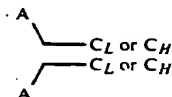
In some embodiments the hybrid immunoglobulins are assembled as monomers or hetero- or homo-multimers, and particularly as dimers or tetramers. Generally, these assembled immunoglobulins will have known unit structures as represented by the following diagrams. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of, basic four-chain units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in a multimeric form in serum. In the case of multimers, each four chain unit may be the same or different.

In the diagrams herein, "A" means at least a portion of a ligand binding partner containing a ligand binding site which is capable of binding its ligand; X is an additional agent, which may be another functional ligand binding partner (same as A or different), a multiple subunit (chain) polypeptide as defined above (e.g., an integrin), a portion of an immunoglobulin superfamily member such as a variable region or a variable region-like domain, including a native or chimeric immunoglobulin variable region, a toxin such as pseudomonas exotoxin or ricin, or a polypeptide therapeutic agent not otherwise normally associated with a constant domain; and V_L, V_H, C_L and C_H represent light or heavy chain variable or constant domains of an immunoglobulin. These diagrams are understood to be merely exemplary of general assembled immunoglobulin structures, and do not encompass all possibilities. It will be understood, for example, that there might desirably be several different "A"s or "X"s in any of these constructs.

monomer:



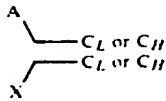
homodimer:



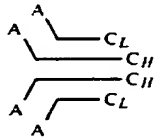
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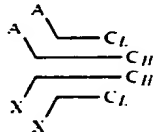
heterodimer:



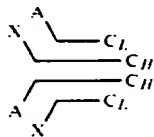
homotetramer:



heterotetramers:



and

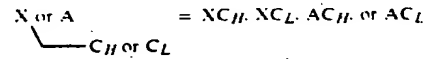


It will be understood that these diagrams are merely illustrative, and that the chains of the multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins. According to this invention, hybrid immunoglobulins are readily secreted from

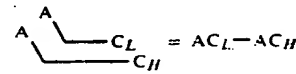
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Chains or basic units of varying structure may be utilized to assemble the monomers and hetero- and homo-multimers and immunoglobulins of this invention. Specific examples of these basic units are diagrammed below and their equivalents (for purposes of the attenuated formulae infra) are indicated.

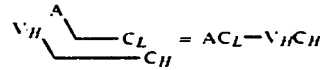
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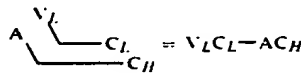
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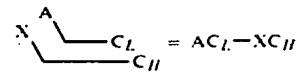
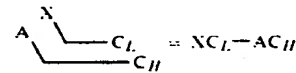
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Various exemplary assembled novel immunoglobulins produced in accordance with this invention are schematically diagrammed below. In addition to the symbols defined above, n is an integer, and Y designates a covalent cross-linking moiety.

- (a) ACL ;
- (b) $ACL-ACL$;
- (c) $AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, V_LC_L-V_HC_H, XC_H, XCL, XC_L-XC_H, XCL-V_HC_H, XC_H-V_LC_L, XCL-AC_H, \text{ or } AC_L-XC_H]$;
- (d) $AC_L-AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, V_LC_L-V_HC_H, XC_H, XCL, XCL-XC_H, XCL-V_HC_H, XC_H-V_LC_L, XCL-AC_H, \text{ or } AC_L-XC_H]$;
- (e) $AC_L-V_HC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, V_LC_L-V_HC_H, XC_H, XCL, XCL-XC_H, XCL-V_HC_H, XC_H-V_LC_L, XCL-AC_H, \text{ or } AC_L-XC_H]$;
- (f) $V_LC_L-AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, V_LC_L-V_HC_H, XC_H, XCL, XCL-XC_H, XCL-V_HC_H, XC_H-V_LC_L, XCL-AC_H, \text{ or } AC_L-XC_H]$;
- (g) $[A-Y]_n-[V_LC_L-V_HC_H]_n$;
- (h) $XC_H \text{ or } XCL-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, XCL-AC_H, \text{ or } AC_L-XC_H]$;
- (i) $XCL-XC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, XCL-AC_H, \text{ or } AC_L-XC_H]$;
- (j) $XCL-V_HC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, XCL-AC_H, \text{ or } AC_L-XC_H]^2$;
- (k) $XC_H-V_LC_L-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, XCL-AC_H, \text{ or } AC_L-XC_H]$;
- (l) $XCL-AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, V_LC_L-V_HC_H, XC_H, XCL, XCL-XC_H, XCL-V_HC_H, XC_H-V_LC_L, XCL-AC_H, \text{ or } AC_L-XC_H]$;
- (m) $AC_L-XC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, V_LC_L-V_HC_H, XC_H, XCL, XCL-XC_H, XCL-V_HC_H, XC_H-V_LC_L, XCL-AC_H, \text{ or } AC_L-XC_H]$;

mammalian cells transformed with the appropriate nucleic acid. The secreted forms include those wherein the binding partner epitope is present in heavy chain dimers, light chain monomers or dimers, and heavy and light chain heterotetramers wherein the binding partner epitope is present fused to one or more light or heavy chains, including heterotetramers wherein up to and including all four variable region analogues are substituted. Where a light-heavy chain non-binding partner variable-like domain is present, a heterofunctional antibody thus is provided.

A, X, V or C may be modified with a covalent cross-linking moiety (Y) so to be $(A-Y)_n$, $(X-Y)_n$, etc.

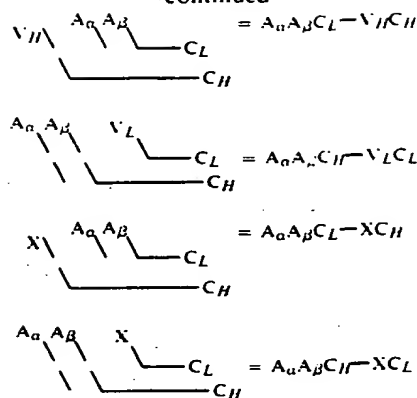
The ligand binding partner A may also be a multi-chain molecule, e.g. having chains arbitrarily denoted as A_α and A_β . These chains as a unit are located at the sites noted for the single chain "A" above. One of the multiple chains is fused to one immunoglobulin chain (with the remaining chains covalently or noncovalently associated with the fused chain in the normal fashion) or, when the ligand binding partner contains two chains, one chain is separately fused to an immunoglob-

ulin light chain and the other chain to an immunoglobulin heavy chain.

It is presently preferred that only one chain of the ligand binding partner be fused to the stable plasma protein. In this case, a fusion through a peptide bond is made between one of the binding partner chains and the stable plasma protein, while the other chain(s) of the ligand binding partner are allowed to associate with the fused chain in the fashion in which they associate in nature, e.g. by disulfide bonding or hydrophobic interaction. The ligand binding partner chain chosen for peptidyl fusion should be the chain which contains a transmembrane domain, and the fusion will be located substantially adjacent N. terminally from the transmembrane domain or in place of the transmembrane and cytoplasmic domains. Ordinarily, if multiple transmembrane domains are present then one is selected for fusion (or deletion and then fusion) while the other remains unfused or is deleted.

Basic units having the structures as diagrammed below are examples of those used to create monomers, and hetero- and homo-multimers, particularly dimers

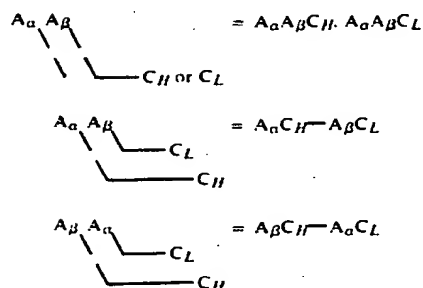
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Various exemplary novel assembled antibodies having a two-chain ligand binding partner ("A_α and A_β") utilized in unit structures as above are schematically diagrammed below.

- (m) A_αA_βC_L-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, V_LC_L-V_HC_H, XC_H, XC_L, XC_L-XC_H, XC_L-V_HC_H, XC_H-V_LC_L, XC_L-AC_H, AC_L-XC_H, A_αA_βC_H, A_αA_βC_L, A_αC_L-A_βC_H, A_βC_L-A_αC_H, A_αA_βC_L-V_HC_H, A_αA_βC_H-V_LC_L, A_αA_βC_L-XC_H, or A_αA_βC_H-XC_L];
- (n) A_αA_βC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, V_LC_L-V_HC_H, XC_H, XC_L, XC_L-XC_H, XC_L-V_HC_H, XC_H-V_LC_L, XC_L-AC_H, AC_L-XC_H, A_αA_βC_H, A_αA_βC_L, A_αC_L-A_βC_H, A_βC_L-A_αC_H, A_αA_βC_L-V_HC_H, A_αA_βC_H-V_LC_L, A_αA_βC_L-XC_H, or A_αA_βC_H-XC_L];
- (p) A_αC_L-A_βC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, V_LC_L-V_HC_H, XC_H, XC_L, XC_L-XC_H, XC_L-V_HC_H, XC_H-V_LC_L, XC_L-AC_H, AC_L-XC_H, A_αA_βC_H, A_αA_βC_L, A_αC_L-A_βC_H, A_βC_L-A_αC_H, A_αA_βC_L-V_HC_H, A_αA_βC_H-V_LC_L, A_αA_βC_L-XC_H, or A_αA_βC_H-XC_L];
- (q) A_βC_L-A_αC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, V_LC_L-V_HC_H, XC_H, XC_L, XC_L-XC_H, XC_L-V_HC_H, XC_H-V_LC_L, XC_L-AC_H, AC_L-XC_H, A_αA_βC_H, A_αA_βC_L, A_αC_L-A_βC_H, A_βC_L-A_αC_H, A_αA_βC_L-V_HC_H, A_αA_βC_H-V_LC_L, A_αA_βC_L-XC_H, or A_αA_βC_H-XC_L];
- (r) A_αA_βC_L-V_HC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, V_LC_L-V_HC_H, XC_H, XC_L, XC_L-XC_H, XC_L-V_HC_H, XC_H-V_LC_L, XC_L-AC_H, AC_L-XC_H, A_αA_βC_H, A_αA_βC_L, A_αC_L-A_βC_H, A_βC_L-A_αC_H, A_αA_βC_L-V_HC_H, A_αA_βC_H-V_LC_L, A_αA_βC_L-XC_H, or A_αA_βC_H-XC_L];
- (s) A_αA_βC_H-V_LC_L-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, V_LC_L-V_HC_H, XC_H, XC_L, XC_L-XC_H, XC_L-V_HC_H, XC_H-V_LC_L, XC_L-AC_H, AC_L-XC_H, A_αA_βC_H, A_αA_βC_L, A_αC_L-A_βC_H, A_βC_L-A_αC_H, A_αA_βC_L-V_HC_H, A_αA_βC_H-V_LC_L, A_αA_βC_L-XC_H, or A_αA_βC_H-XC_L];
- (t) A_αA_βC_L-XC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, V_LC_L-V_HC_H, XC_H, XC_L, XC_L-XC_H, XC_L-V_HC_H, XC_H-V_LC_L, XC_L-AC_H, AC_L-XC_H, A_αA_βC_H, A_αA_βC_L, A_αC_L-A_βC_H, A_βC_L-A_αC_H, A_αA_βC_L-V_HC_H, A_αA_βC_H-V_LC_L, A_αA_βC_L-XC_H, or A_αA_βC_H-XC_L];
- (u) A_αA_βC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, V_LC_L-V_HC_H, XC_H, XC_L, XC_L-XC_H, XC_L-V_HC_H, XC_H-V_LC_L, XC_L-AC_H, AC_L-XC_H, A_αA_βC_H, A_αA_βC_L, A_αC_L-A_βC_H, A_βC_L-A_αC_H, A_αA_βC_L-V_HC_H, A_αA_βC_H-V_LC_L, A_αA_βC_L-XC_H, or A_αA_βC_H-XC_L];

and trimers with multi-chain ligand binding partners:



The structures shown in the above tables show only key features, e.g. they do not show joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. These are omitted in the interests of brevity. However, where such domains are required for binding activity they shall be constructed as being present in the ordinary locations which they occupy in the binding partner or immunoglobulin molecules as the case may be.

Where an immunoglobulin V_LV_H antibody combining site is designated above, or where XC_L or XC_H is indicated and X is an immunoglobulin variable region, it preferably is capable of binding to a predetermined antigen. Suitable immunoglobulin combining sites and fusion partners are obtained from IgG-1, -2, -3, or -4 subtypes, IgA, IgE, IgD or IgM, but preferably IgG-1. The schematic examples above are representative of

divalent antibodies; more complex structures would result by employing immunoglobulin heavy chain sequences from other classes, e.g. IgM.

A particularly preferred embodiment is a fusion of an N-terminal portion of a LHR, which contains the binding site for the endothelium of lymphoid tissue, to the C-terminal Fc portion of an antibody, containing the effector functions of immunoglobulin G₁. There are two preferred embodiments of this sort: in one, the entire heavy chain constant region is fused to a portion of the LHR; in another, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (residue 216, taking the first residue of heavy chain constant region to be 114 [Kabat et al: "Sequences of Proteins of Immunological Interest" 4th Ed: 1987], or analogous sites of other immunoglobulins) is fused to a portion of the LHR. The latter embodiment is described in the Example 4.

Those compositions of this invention, particularly those in which a biologically active portion of a ligand binding partner is substituted for the variable region of an immunoglobulin chain, are believed to exhibit improved in vivo plasma half life. These hybrid immunoglobulins are constructed in a fashion similar to the constructs where a immunoglobulin-like domain is substituted for the variable domain of an immunoglobulin, see e.g. Capon et al: *Nature* 337:525-531, 1989, Trautnecker et al: *Nature* 339:68-70, 1989, Gascoigne et al., *Proc. Nat. Acad. Sci.* 84:2936-2940, 1987, and published European application EPO 0 325 224 A2. The hybrid immunoglobulins of this invention are also constructed in a fashion similar to chimeric antibodies in which a variable domain from an antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; Munro, *Nature* 312: (13 Dec. 1984); Neuberger et al., *Nature* 312: (13 Dec. 1984); Sharon et al: *Nature* 309: (24 May 1984); Morrison et al: *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Morrison et al. *Science* 229:1202-1207 (1985); and Boulianne et al: *Nature* 312:643-646 (13 Dec. 1984). The DNA encoding the binding partner is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the desired binding partner domain(s) and at a point at or near the DNA encoding the N-terminal end of the mature polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for the binding partner (where a native signal is employed). This DNA fragment then is readily inserted into DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, tailored by deletional mutagenesis. Preferably, this is a human immunoglobulin when the variant is intended for in vivo therapy for humans.

The LHR extracellular domain generally is fused at its C-terminus to the immunoglobulin constant region. The precise site at which the fusion is made is not critical, other sites neighboring or within the extracellular region may be selected in order to optimize the secretion or binding characteristics of the soluble LHR-Ig fusion. The optimal site will be determined by routine experimentation. The fusion may typically take the place of either or both the transmembrane and cytoplasmic domains.

DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams et al., *Biochemistry* 19:2711-2719 (1980); Gough

et al., *Biochemistry* 19:2702-2710 (1980); Dolby et al: *P.N.A.S. USA*, 77:6027-6031 (1980); Rice et al *P.N.A.S. USA* 79:7862-7865 (1982); Falkner et al: *Nature* 298:286-288 (1982); and Morrison et al: *Ann. Rev. Immunol.* 2:239-256 (1984). DNA sequences encoding the LHR are provided herein. DNA sequences encoding other desired binding partners which are known or readily available from cDNA libraries are suitable in the practice of this invention.

DNA encoding a fusion of this invention is transfected into a host cell for expression. If multimers are desired then the host cell is transformed with DNA encoding each chain that will make up the multimer, with the host cell optimally being selected to be capable of assembling the chains of the multimers in the desired fashion. If the host cell is producing an immunoglobulin prior to transfection then one need only transfect with the binding partner fused to light or to heavy chain to produce a heteroantibody. The aforementioned immunoglobulins having one or more arms bearing the binding partner domain and one or more arms bearing companion variable regions result in dual specificity for the binding partner ligand and for an antigen or therapeutic moiety. Multiply cotransformed cells are used with the above-described recombinant methods to produce polypeptides having multiple specificities such as the heterotetrameric immunoglobulins discussed above.

In general, it has been found that the fusions are expressed intracellularly and well secreted, but a great deal of variation is routinely encountered in the degree of secretion of various fusions from recombinant hosts.

Additionally, procedures are known for producing intact heteroantibodies from immunoglobulins having different specificities. These procedures are adopted for the in vitro synthesis or production of heterochimeric antibodies by simply substituting the binding partner-immunoglobulin chains where an immunoglobulin or immunoglobulin hybrid was previously used.

In an alternative method for producing a heterofunctional antibody, host cells producing a binding partner-immunoglobulin fusion, e.g. transfected myelomas, also are fused with B cells or hybridomas which secrete antibody having the desired companion specificity for an antigen. Heterobifunctional antibody is recovered from the culture medium of such hybridomas, and thus may be produced somewhat more conveniently than by conventional in vitro resorting methods (EP 68,763).

This invention also contemplates amino acid sequence variants of the LHR or other binding partner. Amino acid sequence variants of the binding partner are prepared with various objectives in mind, including increasing the affinity of the binding partner for its ligand, facilitating the stability, purification and preparation of the binding partner, modifying its plasma half life, improving therapeutic efficacy and lessening the severity or occurrence of side effects during therapeutic use of the binding partner. In the discussion below, amino acid sequence variants of the LHR are provided exemplary of the variants that may be selected for other ligand binding partners.

Amino acid sequence variants of the ligand binding partner fall into one or more of three classes: Insertional, substitutional, or deletional variants. These variants ordinarily are prepared by site-specific mutagenesis of nucleotides in the DNA encoding the ligand binding partner, by which DNA encoding the variant is obtained, and thereafter expressing the DNA in recombinant cell culture. However, fragments having up to

about 100-150 amino acid residues are prepared conveniently by *in vitro* synthesis. While the following discussion in part refers to LHR, it applies with equal effect to any ligand binding partner to the extent it is applicable to the structure or function thereof.

The amino acid sequence variants of the LHR are predetermined variants not found in nature or naturally occurring alleles. The LHR variants typically exhibit the same qualitative biological—for example, ligand binding—activity as the naturally occurring HuLHR or MLHR analogue. However, the LHR variants and derivatives that are not capable of binding to their ligands are useful nonetheless (a) as a reagent in diagnostic assays for the LHR or antibodies to the LHR, (b) when insolubilized in accord with known methods, as agents for purifying anti-LHR antibodies from antisera or hybridoma culture supernatants, and (c) as immunogens for raising antibodies to the LHR or as immunoassay kit components (labelled as a competitive reagent for the native LHR or unlabelled as a standard for the LHR assay) so long as at least one LHR epitope remains active.

While the site for introducing an amino acid sequence variation is predetermined, the mutation *per se* need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random or saturation mutagenesis (where all 20 possible residues are inserted) is conducted at the target codon and the expressed LHR variant is screened for the optimal combination of desired activities. Such screening is within the ordinary skill in the art.

Amino acid insertions usually will be on the order of about from 1 to 10 amino acid residues; substitutions are typically introduced for single residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. It will be amply apparent from the following discussion that substitutions, deletions, insertions or any combination thereof are introduced or combined to arrive at a final construct.

Insertional amino acid sequence variants of the LHR are those in which one or more amino acid residues extraneous to the LHR are introduced into a predetermined site in the target LHR and which displace the preexisting residues.

Commonly, insertional variants are fusions of heterologous proteins or polypeptides to the amino or carboxyl terminus of the LHR. Such variants are referred to as fusions of the LHR and a polypeptide containing a sequence which is other than that which is normally found in the LHR at the inserted position. Several groups of fusions are contemplated herein.

The novel polypeptides of this invention are useful in diagnostics or in purification of the ligand binding partner by immunoaffinity techniques known *per se*. Alternatively, in the purification of the binding partner, the novel polypeptides are used to adsorb the fusion from impure admixtures, after which the fusion is eluted and, if desired, the binding partner is recovered from the fusion, e.g. by enzymatic cleavage.

Desirable fusions of the binding partner, which may or may not also be immunologically active, include fusions of the mature binding partner sequence with a signal sequence heterologous to the binding partner.

In the case of the LHR, and where desired with other selected binding proteins, signal sequence fusions are employed in order to more expeditiously direct the

secretion of the LHR. The heterologous signal replaces the native LHR signal, and when the resulting fusion is recognized, i.e. processed and cleaved by the host cell, the LHR is secreted. Signals are selected based on the intended host cell, and may include bacterial yeast, mammalian and viral sequences. The native LHR signal or the herpes gD glycoprotein signal is suitable for use in mammalian expression systems.

Substitutional variants are those in which at least one residue in the FIG. 1 or 2 sequence has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Table 1 when it is desired to finely modulate the characteristics of the LHR.

TABLE 1

Original Residue	Exemplary Substitutions
Ala	ser
Arg	lys
Asn	gln; his
Asp	glu
Cys	ser; ala
Gln	asn
Glu	asp
Gly	pro
His	asn; gln
Ile	leu; val
Leu	ile; val
Lys	arg; gln; glu
Met	leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

Novel amino acid sequences, as well as isosteric analogs (amino acid or otherwise), as included within the scope of this invention.

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 1, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in LHR properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine.

Some deletions, insertions, and substitutions will not produce radical changes in the characteristics of the LHR molecule. However, when it is difficult to predict the exact effect of the substitution, deletion or insertion in advance of doing so, for example when modifying the LHR carbohydrate binding domain or an immune epitope, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. For example, a variant typically is made by site specific mutagenesis of the LHR-encoding nucleic acid, expression of the variant nucleic acid in recombinant cell culture

and, optionally, purification from the cell culture for example by immunoaffinity adsorption on a polyclonal anti-LHR column (in order to adsorb the variant by at least one remaining immune epitope). The activity of the cell lysate or purified LHR variant is then screened in a suitable screening assay for the desired characteristic. For example, a change in the immunological character of the LHR, such as affinity for a given antibody such as Mel-14, is measured by a competitive-type immunoassay. As more becomes known about the functions in vivo of the LHR other assays will become useful in such screening. Modifications of such protein properties as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, or the tendency to aggregate with carriers or into multimers are assayed by methods well known to the artisan.

Substitutional variants of the LHR also include variants where functionally homologous domains of other proteins are substituted by routine methods for one or more of the above-identified LHR domains. Where the variant is a fragment of a particular domain of the LHR, it preferably but not necessarily has at least ~70% homology to the corresponding LHR domain as defined herein. FIGS. 6A-6C may be used by those skilled in the art for sources for such substitutable domains. For example, the flesh fly lectin whose sequence is shown in FIG. 6A may be modified to rise to the level of at least ~70% homology with the carbohydrate binding domain of the LHR, and then substituted for that domain. Similarly, coagulation Factor X, whose sequence is shown in FIG. 6B may be modified to rise to the level of at least ~70% homology with the egf-domain of the LHR, and then substituted for that domain. Similar substitutions may desirably be made for the signal sequence, the complement binding domain, the transmembrane domain, and for the cytoplasmic domain.

Another class of LHR variants are deletional variants. Deletions are characterized by the removal of one or more amino acid residues from the LHR sequence. Typically, the transmembrane and cytoplasmic domains, or only the cytoplasmic domains of the LHR are deleted. However, deletion from the LHR C-terminal to any other suitable site N-terminal to the transmembrane region which preserves the biological activity or immune cross-reactivity of the LHR is suitable. Excluded from the scope of deletional variants are the protein digestion fragments heretofore obtained in the course of elucidating amino acid sequences of the LHR, and protein fragments having less than ~70% sequence homology to any of the above-identified LHR domains.

Immunoglobulin fusions may be made with fragments of the LHR, such as the complement binding domain, the carbohydrate domain, and the epidermal growth factor domain. The complement binding domain fusion finds usefulness in the diagnosis and treatment of complement-mediated diseases, as well as in the oligomerization of the fusion with the LHR or with other components on the lymphocyte surface.

Deletions of cysteine or other labile residues also may be desirable, for example in increasing the oxidative stability of the LHR. Deletion or substitutions of potential proteolysis sites, e.g. Arg Arg, is accomplished by deleting one of the basic residues or substituting one by glutamyl or histidyl residues.

In one embodiment, the LHR is comprised of the carbohydrate binding domain in the absence of a complement binding domain and/or the egf domain. This

embodiment may or may not contain either or both the transmembrane and cytoplasmic regions.

A preferred class of substitutional or deletional variants are those involving a transmembrane region of the LHR. Transmembrane regions of LHR subunits are highly hydrophobic or lipophilic domains that are the proper size to span the lipid bilayer of the cellular membrane. They are believed to anchor the LHR in the cell membrane, and allow for homo- or heteropolymeric complex formation with the LHR.

Inactivation of the transmembrane domain of the LHR and any other binding partner where one is present, typically by deletion or substitution of transmembrane domain hydroxylation residues, will facilitate recovery and formulation by reducing its cellular or membrane lipid affinity and improving its aqueous solubility. If the transmembrane and cytoplasmic domains are deleted one avoids the introduction of potentially immunogenic epitopes, either by exposure of otherwise intracellular polypeptides that might be recognized by the body as foreign or by insertion of heterologous polypeptides that are potentially immunogenic. Inactivation of the membrane binding function is accomplished by deletion of sufficient residues to produce a substantially hydrophilic hydropathy profile at this site or by substituting with heterologous residues which accomplish the same result.

A principal advantage of the transmembrane inactivated LHR is that it may be secreted into the culture medium of recombinant hosts. This variant is soluble in body fluids such as blood and does not have an appreciable affinity for cell membrane lipids, thus considerably simplifying its recovery from recombinant cell culture.

As a general proposition, all variants will not have a functional transmembrane domain and preferably will not have a functional cytoplasmic sequence.

For example, the transmembrane domain may be substituted by any amino acid sequence, e.g. a random or predetermined sequence of about 5 to 50 serine, threonine, lysine, arginine, glutamine, aspartic acid and like hydrophilic residues, which altogether exhibit a hydrophilic hydropathy profile. Like the deletional (truncated) LHR, these variants are secreted into the culture medium of recombinant hosts.

Examples of HuLHR amino acid sequence variants are described in the table below. The residue following the residue number indicates the replacement or inserted amino acids.

TABLE 2

Substitutions	Deletions	Insertions
Arg58—Asp59: Lys—Glu	Gly96—Ile97	67-Glu—Ser—Ala
Ala71: Ser	Asn136	83-Gly—Thr—Thr
Lys78: Gln	Ser166	209-Asn
Asp116: Glu	Ser220	241-Val—Glu—Asn
Leu150: Val	Asn271	292-Tyr—Tyr—Tyr
His168: Gln	Ile296	
Ile174: Leu		
Asn181: Gln		
Thr211: Ser		
Phe214: Leu		
Ser226: Thr		
Phe244: Met		
Thr282: Ser		
Ile288: Val		
Lys298—Lys299: Arg—Arg		
Ile302: Leu		

Preferably, the variants represent conservative substitutions. It will be understood that some variants may exhibit reduced or absent biological activity. These variants nonetheless are useful as standards in immunoassays for the LHR so long as they retain at least one immune epitope of the LHR.

Glycosylation variants are included within the scope of the HuLHR. They include variants completely lacking in glycosylation (unglycosylated) and variants having at least one less glycosylated site than the native form (deglycosylated) as well as variants in which the glycosylation has been changed. Included are deglycosylated and unglycosylated amino acid sequence variants, deglycosylated and unglycosylated LHR having the native, unmodified amino acid sequence of the LHR, and other glycosylation variants. For example, substitutional or deletional mutagenesis is employed to eliminate the N- or O-linked glycosylation sites of the LHR, e.g. the asparagine residue is deleted or substituted for by another basic residue such as lysine or histidine. Alternatively, flanking residues making up the glycosylation site are substituted or deleted, even though the asparagine residues remain unchanged, in order to prevent glycosylation by eliminating the glycosylation recognition site.

Additionally, unglycosylated LHR which has the amino acid sequence of the native LHR is produced in recombinant prokaryotic cell culture because prokaryotes are incapable of introducing glycosylation into polypeptides.

Glycosylation variants are produced by selecting appropriate host cells or by in vitro methods. Yeast, for example, introduce glycosylation which varies significantly from that of mammalian systems. Similarly, mammalian cells having a different species (e.g. hamster, murine, insect, porcine, bovine or ovine) or tissue origin (e.g. lung, liver, lymphoid, mesenchymal or epidermal) than the source of the LHR are routinely screened for the ability to introduce variant glycosylation as characterized for example by elevated levels of mannose or variant ratios of mannose, fucose, sialic acid, and other sugars typically found in mammalian glycoproteins. In vitro processing of the LHR typically is accomplished by enzymatic hydrolysis, e.g. neuraminidase digestion.

Covalent modifications of the LHR molecule are included within the scope hereof. Such modifications are introduced by reacting targeted amino acid residues of the recovered protein with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues, or by harnessing mechanisms of post-translational modification that function in selected recombinant host cells. The resulting covalent derivatives are useful in programs directed at identifying residues important for biological activity, for immunoassays of the LHR or for the preparation of anti-LHR antibodies for immunoaffinity purification of the recombinant LHR. For example, complete inactivation of the biological activity of the protein after reaction with ninhydrin would suggest that at least one arginyl or lysyl residue is critical for its activity, whereafter the individual residues which were modified under the conditions selected are identified by isolation of a peptide fragment containing the modified amino acid residue. Such modifications are within the ordinary skill in the art and are performed without undue experimentation.

Derivatization with bifunctional agents is useful for preparing intermolecular aggregates of the hybrid immunoglobulin with polypeptides as well as for cross-linking the hybrid immunoglobulin to a water insoluble support matrix or surface for use in the assay or affinity purification of its ligands. In addition, a study of intrachain cross-links will provide direct information on conformational structure. Commonly used cross-linking agents include 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example esters with 4-azidosalicylic acid, homobifunctional imidoesters including disuccinimidyl esters such as 3,3'-dithiobis (succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)-dithio]propionimidate yield photoactivatable intermediates which are capable of forming cross-links in the presence of light. Alternatively, reactive water insoluble matrices such as cyanogen bromide activated carbohydrates and the systems reactive substrates described in U.S. Pat. Nos. 3,959,080; 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; 4,055,635; and 4,330,440 are employed for protein immobilization and cross-linking.

Certain post-translational derivatizations are the result of the action of recombinant host cells on the expressed polypeptide. Glutamyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, *Proteins Structure and Molecular Properties*, W. H. Freeman & Co: San Francisco pp 79-86 [1983]), acetylation of the N-terminal amine and, in some instances, amidation of the C-terminal carboxyl.

Other derivatives comprise the novel polypeptides of this invention covalently bonded to a nonproteinaceous polymer. The nonproteinaceous polymer ordinarily is a hydrophilic synthetic polymer, i.e., a polymer not otherwise found in nature. However polymers which exist in nature and are produced by recombinant or in vitro methods are useful, as are polymers which are isolated from nature. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyalkylene ethers such as polyethylene glycol, polypropylene glycol, polyoxyethylene esters or methoxy polyethylene glycol; polyoxyalkylenes such as polyoxyethylene, polyoxypropylene, and block copolymers of polyoxyethylene and polyoxypropylene (Pluronic); polymethacrylates; carbomers; branched or unbranched polysaccharides which comprise the saccharide monomers D-mannose, D- and L-galactose, fucose, fructose, D-xylose, L-arabinose, D-glucuronic acid, sialic acid, D-galacturonic acid, D-mannuronic acid (e.g. polymanuronic acid, or alginic acid), D-glucosamine, D-galactosamine, D-glucose and neuraminic acid including homopolysaccharides and heteropolysaccharides such as lactose, amylopectin, starch, hydroxyethyl starch, amylose, dextran sulfate, dextran, dextrans glycogen, or the polysaccharide subunit of acid mucopoly, saccharides, e.g. hyaluronic acid; polymers of sugar alcohols

such as polysorbitol and polymannitol; and heparin or heparan.

Where the polysaccharide is the native glycosylation or the glycosylation attendant on recombinant expression, the site of substitution may be located at other than a native N- or O-linked glycosylation site wherein an additional or substitute N or O-linked site has been introduced into the molecule. Mixtures of such polymers may be employed, or the polymer may be homogeneous. The polymer prior to crosslinking need not be, but preferably is, water soluble, but the final conjugate must be water soluble. In addition, the polymer should not be highly immunogenic in the conjugate form, nor should it possess viscosity that is incompatible with intravenous infusion or injection if it is intended to be administered by such routes.

Preferably the polymer contains only a single group which is reactive. This helps to avoid cross-linking of protein molecules. However, it is within the scope herein to optimize reaction conditions to reduce cross-linking, or to purify the reaction products through gel filtration or chromatographic sieves to recover substantially homogeneous derivatives.

The molecular weight of the polymer may desirably range from about 100 to 500,000, and preferably is from about 1,000 to 20,000. The molecular weight chosen will depend upon the nature of the polymer and the degree of substitution. In general the greater the hydrophilicity of the polymer and the greater the degree of substitution, the lower the molecular weight that can be employed. Optimal molecular weights will be determined by routine experimentation.

The polymer generally is covalently linked to the hybrid immunoglobulin herein through a multifunctional crosslinking agent which reacts with the polymer and one or more amino acid or sugar residues of the hybrid. However, it is within the scope of this invention to directly crosslink the polymer by reacting a derivatized polymer with the hybrid, or vice versa.

The covalent crosslinking site on the hybrid immunoglobulin includes the N-terminal amino group and epsilon amino groups found on lysine residues, as well as other amino, imino, carboxyl, sulfhydryl, hydroxyl or other hydrophilic groups. The polymer may be covalently bonded directly to the hybrid without the use of a multifunctional (ordinarily bifunctional) crosslinking agent. Covalent bonding to amino groups is accomplished by known chemistries based upon cyanuric chloride, carbonyl diimidazole, aldehyde reactive groups (PEG alkoxide plus diethyl acetal of bromoacetaldehyde; PEG plus DMSO and acetic anhydride, or PEG chloride plus the phenoxide of 4-hydroxybenzaldehyde, succinimidyl active esters, activated dithiocarbonate PEG, 2,4,5-trichlorophenylchloroformate or p-nitrophenylchloroformate activated PEG. Carboxyl groups are derivatized by coupling PEG-amine using carbodiimide.

Polymers are conjugated to oligosaccharide groups by oxidation using chemicals, e.g. metaperiodate, or enzymes, e.g. glucose or galactose oxidase, (either of which produces the aldehyde derivative of the carbohydrate), followed by reaction with hydrazide or amino-derivatized polymers, in the same fashion as is described by Heitzmann et al., P.N.A.S. 71:3537-3541 (1974) or Bayer et al; Methods in Enzymology, 62:310 (1979), for the labeling of oligosaccharides with biotin or avidin. Further, other chemical or enzymatic methods which have been used heretofore to link oligosaccharides and

polymers are suitable. Substituted oligosaccharides are particularly advantageous because, in general, there are fewer substitutions than amino acid sites for derivatization, and the oligosaccharide products thus will be more homogeneous. The oligosaccharide substituents also are optionally modified by enzyme digestion to remove sugars, e.g. by neuraminidase digestion, prior to polymer derivatization.

The polymer will bear a group which is directly reactive with an amino acid side chain, or the N- or C-terminus of the polypeptide herein, or which is reactive with the multifunctional cross-linking agent. In general, polymers bearing such reactive groups are known for the preparation of immobilized proteins. In order to use such chemistries here, one should employ a water soluble polymer otherwise derivatized in the same fashion as insoluble polymers heretofore employed for protein immobilization. Cyanogen bromide activation is a particularly useful procedure to employ in crosslinking polysaccharides.

"Water soluble" in reference to the starting polymer means that the polymer or its reactive intermediate used for conjugation is sufficiently water soluble to participate in a derivatization reaction.

"Water soluble" in reference to the polymer conjugate means that the conjugate is soluble in physiological fluids such as blood.

The degree of substitution with such a polymer will vary depending upon the number of reactive sites on the protein, whether all or a fragment of the protein is used, whether the protein is a fusion with a heterologous protein, the molecular weight, hydrophilicity and other characteristics of the polymer, and the particular protein derivatization sites chosen. In general, the conjugate contains about from 1 to 10 polymer molecules, while any heterologous sequence may be substituted with an essentially unlimited number of polymer molecules so long as the desired activity is not significantly adversely affected. The optimal degree of crosslinking is easily determined by an experimental matrix in which the time, temperature and other reaction conditions are varied to change the degree of substitution, after which the ability of the conjugates to function in the desired fashion is determined.

The polymer, e.g. PEG, is crosslinked by a wide variety of methods known per se for the covalent modification of proteins with nonproteinaceous polymers such as PEG. Certain of these methods, however, are not preferred for the purposes herein. Cyanuric chloride chemistry leads to many side reactions, including protein cross-linking. In addition, it may be particularly likely to lead to inactivation of proteins containing sulfhydryl groups. Carbonyl diimidazole chemistry (Beauchamp et al., "Anal. Biochem." 131:25-33 [1983]) requires high pH (>8.5), which can inactivate proteins. Moreover, since the "activated PEG" intermediate can react with water, a very large molar excess of "activated PEG" over protein is required. The high concentrations of PEG required for the carbonyl diimidazole chemistry also led to problems with purification, as both gel filtration chromatography and hydrophobic interaction chromatography are adversely effected. In addition, the high concentrations of "activated PEG" may precipitate protein, a problem that per se has been noted previously (Davis, U.S. Pat. No. 4,179,337). On the other hand, aldehyde chemistry (Royer, U.S. Pat. No. 4,002,531) is more efficient since it requires only a 40 fold molar excess of PEG and a 1-2 hr incubation.

However, the manganese dioxide suggested by Royer for preparation of the PEG aldehyde is problematic "because of the pronounced tendency of PEG to form complexes with metal-based oxidizing agents" (Harris et al. "J. Polym. Sci.: Polym. Chem. Ed." 22:341-352 [1984]). The use of a Moffatt oxidation, utilizing DMSO and acetic anhydride, obviates this problem. In addition, the sodium borohydride suggested by Royer must be used at a high pH and has a significant tendency to reduce disulfide bonds. In contrast, sodium cyanoborohydride, which is effective at neutral pH and has very little tendency to reduce disulfide bonds is preferred.

The conjugates of this invention are separated from unreacted starting materials by gel filtration. Heterologous species of the conjugates are purified from one another in the same fashion.

The polymer also may be water insoluble, as a hydrophilic gel or a shaped article such as surgical tubing in the form of catheters or drainage conduits.

DNA encoding the LHR and other ligand binding partners is synthesized by in vitro methods or is obtained readily from lymphocyte cDNA libraries. The means for synthetic creation of the DNA encoding the LHR, either by hand or with an automated apparatus, are generally known to one of ordinary skill in the art, particularly in light of the teachings contained herein. As examples of the current state of the art relating to polynucleotide synthesis, one is directed to Maniatis et al., *Molecular Cloning—Laboratory Manual*, Cold Spring Harbor Laboratory (1984), and Horvath et al., *An Automated DNA Synthesizer Employing Deoxynucleoside 3'-Phosphoramidites*, *Methods in Enzymology* 154: 313-326, 1987, hereby specifically incorporated by reference.

Alternatively, to obtain DNA encoding the LHR from sources other than murine or human, since the entire DNA sequence for the preferred embodiment of the HuLHR (FIG. 1) and of the MLHR (FIG. 2) are given, one needs only to conduct hybridization screening with labelled DNA encoding either HuLHR or MLHR or fragments thereof (usually, greater than about 20, and ordinarily about 50 bp) in order to detect clones which contain homologous sequences in the cDNA libraries derived from the lymphocytes of the particular animal, followed by analyzing the clones by restriction enzyme analysis and nucleic acid sequencing to identify full length clones. If full length clones are not present in the library, then appropriate fragments are recovered from the various clones and ligated at restriction sites common to the fragments to assemble a full-length clone. DNA encoding the LHR from other animal species is obtained by probing libraries from such species with the human or murine sequences, or by synthesizing the genes in vitro. DNA for other binding partners having known sequence may be obtained with the use of analogous routine hybridization procedures.

Provided herein are nucleic acid sequences that hybridize under stringent conditions to a fragment of the DNA sequence in FIG. 1 or FIG. 2, which fragment is greater than about 10 bp, preferably 20-50 bp, and even greater than 100 bp. Also included within the scope hereof are nucleic acid sequences that hybridize under stringent conditions to a fragment of the LHR other than the signal, or transmembrane, or cytoplasmic domains.

Included also within the scope hereof are nucleic acid probes which are capable of hybridizing under stringent conditions to the cDNA of the LHR or to the genomic

gene for the LHR (including introns and 5' or 3' flanking regions extending to the adjacent genes or about 5,000 bp, whichever is greater).

Identification of the genomic DNA for the LHR or other binding partner is a straight-forward matter of probing a particular genomic library with the cDNA or its fragments which have been labelled with a detectable group, e.g. radiophosphorus, and recovering clone(s) containing the gene. The complete gene is pieced together by "walking" if necessary. Typically, such probes do not encode sequences with less than 70% homology to HuLHR or MLHR, and they range from about 10 to 100 bp in length. Homologies and sizes with respect to other binding partners may be determined without undue experimentation.

In general, prokaryotes are used for cloning of DNA sequences in constructing the vectors useful in the invention. For example, *E. coli* K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include *E. coli* B and *E. coli* X1776 (ATCC No. 31537). These examples are illustrative rather than limiting. Alternatively, in vitro methods of cloning, e.g. polymerase chain reaction, are suitable.

The polypeptides of this invention are expressed directly in recombinant cell culture as an N-terminal methionyl analogue, or as a fusion with a polypeptide heterologous to the hybrid/portion, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the hybrid/portion. For example, in constructing a prokaryotic secretory expression vector for the LHR, the native LHR signal is employed with hosts that recognize that signal. When the secretory leader is "recognized" by the host, the host signal peptidase is capable of cleaving a fusion of the leader polypeptide fused at its G-terminus to the desired mature LHR. For host prokaryotes that do not process the LHR signal, the signal is substituted by a prokaryotic signal selected for example from the group of the alkaline phosphatase, penicillinase, lpp or heat stable enterotoxin II leaders. For yeast secretion the human LHR signal may be substituted by the yeast invertase, alpha factor or acid phosphatase leaders. In mammalian cell expression the native signal is satisfactory for mammalian LHR, although other mammalian secretory protein signals are suitable, as are viral secretory leaders, for example the herpes simplex gD signal.

The novel polypeptides may be expressed in any host cell, but preferably are synthesized in mammalian hosts. However, host cells from prokaryotes, fungi, yeast, insects and the like are also used for expression. Exemplary prokaryotes are the strains suitable for cloning as well as *E. coli* W3110 (F⁻, λ^{-} , prototrophic, ATCC No. 27325), other enterobacteriaceae such as *Serratia marcescens*, bacilli and various pseudomonads. Preferably the host cell should secrete minimal amounts of proteolytic enzymes.

Expression hosts typically are transformed with DNA encoding the hybrid which has been ligated into an expression vector. Such vectors ordinarily carry a replication site (although this is not necessary where chromosomal integration will occur). Expression vectors also include marker sequences which are capable of providing phenotypic selection in transformed cells, as will be discussed further below. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar, et al., *Gene* 2: 95 [1977]). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for

identifying transformed cells, whether for purposes of cloning or expression. Expression vectors also optimally will contain sequences which are useful for the control of transcription and translation, e.g. promoters and Shine-Dalgarno sequences (for prokaryotes) or promoters and enhancers (for mammalian cells). The promoters may be, but need not be, inducible; surprisingly, even powerful constitutive promoters such as the CMV promoter for mammalian hosts have been found to produce the LHR without host cell toxicity. While it is conceivable that expression vectors need not contain any expression control, replicative sequences or selection genes, their absence may hamper the identification of hybrid transformants and the achievement of high level hybrid immunoglobulin expression.

Promoters suitable for use with prokaryotic hosts illustratively include the β -lactamase and lactose promoter systems (Chang et al., "Nature", 275: 615 [1978]; and Goeddel et al., "Nature" 281: 544 [1979]), alkaline phosphatase, the tryptophan (trp) promoter system (Goeddel "Nucleic Acids Res." 8: 4057 [1980] and EPO Appln. Publ. No. 36,776) and hybrid promoters such as the tac promoter (H. de Boer et al., "Proc. Natl. Acad. Sci. USA" 80: 21-25 [1983]). However, other functional bacterial promoters are suitable. Their nucleotide sequences are generally known, thereby enabling a skilled worker operably to ligate them to DNA encoding the LHR (Siebenlist et al., "Cell" 20:269 [1980]) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the LHR.

In addition to prokaryotes, eukaryotic microbes such as yeast or filamentous fungi are satisfactory. *Saccharomyces cerevisiae* is the most commonly used eukaryotic microorganism, although a number of other strains are commonly available. The plasmid YRp7 is a satisfactory expression vector in yeast (Stinchcomb, et al., Nature 282: 39 [1979]; Kingsman et al. Gene 7: 141 [1979]; Tschemper et al. Gene 10: 157 [1980]). This plasmid already contains the trp1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC no. 44076 or PEP4-1 (Jones, Genetics 85: 12 [1977]). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., "J. Biol. Chem." 255: 2073 [1980]) or other glycolytic enzymes (Hess et al., "J. Adv. Enzyme Reg." 7: 149 [1968]; and Holland, "Biochemistry" 17: 4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further

described in R. Hitzeman et al., European Patent Publication No. 73,657A.

Expression control sequences are known for eucaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence which may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are inserted into mammalian expression vectors.

Suitable promoters for controlling transcription from vectors in mammalian host cells are readily obtained from various sources, for example, the genomes of viruses such as polyoma virus, SV40, adenovirus, MMV (steroid inducible), retroviruses (e.g. the LTR of HIV), hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. the beta actin promoter. The early and late promoters of SV40 are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication. Fiers et al., Nature, 273: 113 (1978). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway, P. J. et al., Gene 18: 355-360 (1982).

Transcription of a DNA encoding the hybrid immunoglobulin and/or hybrid portions by higher eucaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10-300bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' (Laimins, L. et al., PNAS 78: 993 [1981]) and 3' (Lusky, M. L., et al., Mol. Cell Bio. 3: 1108 [1983]) to the transcription unit, within an intron (Banerji, J. L. et al., Cell 33: 729 [1983]) as well as within the coding sequence itself (Osborne, T. F., et al., Mol. Cell Bio. 4: 1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding the hybrid immunoglobulin. The 3' untranslated regions also include transcription termination sites.

Expression vectors may contain a selection gene, also termed a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase (TK) or neomycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell is able to survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a

supplemented media. Two examples are CHO DHFR - cells and mouse LTK - cells. These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non supplemented media.

The second category of selective regimes is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern et al., *J. Molec. Appl. Genet.* 1: 327 (1982)), mycophenolic acid (Mulligan et al., *Science* 209: 1422 (1980)) or hygromycin (Sugden et al., *Mol. Cell. Biol.* 5: 410-413 (1985)). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), *agpt* (mycophenolic acid) or hygromycin, respectively.

Suitable eukaryotic host cells for expressing the hybrid immunoglobulin include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham, F. L. et al., *J. Gen. Virol.* 36: 59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); chinese hamster ovary-cells-DHFR (CHO, Urlaub and Chasin, *PNAS (USA)* 77: 4216, [1980]); mouse sertoli cells (TM4, Mather, J. P., *Biol. Reprod.* 23: 243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); african green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); and, TR1 cells (Mather, J. P. et al., *Annals N.Y. Acad. Sci.* 383: 44-68 [1982]).

Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction and/or sequenced by the method of Messing et al., *Nucleic Acids Res.* 9: 309 (1981) or by the method of Maxam et al., *Methods in Enzymology* 65: 499 (1980).

Host cells are transformed with the expression vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants or amplifying the genes encoding the desired sequences. The culture conditions, such as temperature, pH and the like, are those previ-

ously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in *in vitro* culture as well as cells which are within a host animal.

"Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integration. Unless indicated otherwise, the method used herein for transformation of the host cells is the method of Graham, F. and van der Eb, A., *Virology* 52: 456-457 (1973). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used. If prokaryotic cells or cells which contain substantial cell wall constructions are used, the preferred method of transfection is calcium treatment using calcium chloride as described by Cohen, F. N. et al., *Proc. Natl. Acad. Sci. (USA)*, 69: 2110 (1972).

"Transfection" refers to the introduction of DNA into a host cell whether or not any coding sequences are ultimately expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO_4 and electro-poration. Transformation of the host cell is the indicia of successful transfection.

The novel polypeptide is recovered and purified from recombinant cell cultures by known methods, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, immunoaffinity chromatography, hydroxyapatite chromatography and lectin chromatography. Other known purification methods within the scope of this invention utilize immobilized carbohydrates, epidermal growth factor, or complement domains. Moreover, reverse-phase HPLC and chromatography using ligands for the hybrid immunoglobulin are useful for the purification of the hybrid. Desirably, low concentrations (approximately 1-5 mM) of calcium ion may be present during purification. The LHR may preferably be purified in the presence of a protease inhibitor such as PMSF.

The LHR-immunoglobulin hybrid is employed therapeutically to compete with the normal binding of lymphocytes to lymphoid tissue. The hybrid is therefore particularly useful for organ or graft rejection, and for the treatment of patients with inflammations, such as are for example due to rheumatoid arthritis or other autoimmune diseases. The LHR-immunoglobulin hybrid also finds application in the control of lymphoma metastasis, and in treating conditions in which there is an accumulation of lymphocytes.

LHR-immunoglobulin hybrid heterodimers and heterotetramers are employed in the targeting of therapeutic moieties to lymphoid tissues. For example, a hybrid immunoglobulin consisting of one LHR-IgG chain and one CD4-IgG chain can be used to target CD4 IgG to tissues infected by the viruses such as the human immunodeficiency virus (HIV). Because this hybrid binds to endothelial tissue not only in lymph nodes, but in secondary lymphoid organs such as Peyer's patches and in the brain, it may be used for delivery of CD4-IgG across the blood-brain barrier for the treatment of HIV-related dementia. Similarly, a heterotetrameric immunoglobulin having a LHR-ricin or CD4-ricin-immunoglobulin as described herein is used to deliver a toxin such as ricin to desired tissues.

In this fashion, selection of ligand binding partners with specific affinity for particular tissues clearly en-

hances the ability to deliver therapeutic agents which are stable, have relatively long half-lives, and are capable of precise tailoring without undue experimentation.

The novel polypeptide is placed into sterile, isotonic formulations together with required cofactors, and optionally are administered by standard means well known in the field. The formulation is preferably liquid, and is ordinarily a physiologic salt solution containing 0.5-10 mM calcium, non-phosphate buffer at pH 6.8-7.6, or may be lyophilized powder.

It is envisioned that intravenous delivery, or delivery through catheter or other surgical tubing will be the primary route for therapeutic administration. Alternative routes include tablets and the like, commercially available nebulizers for liquid formulations, and inhalation of lyophilized or aerosolized receptors. Liquid formulations may be utilized after reconstitution from powder formulations.

The novel polypeptide may also be administered via microspheres, liposomes, other microparticulate delivery systems or sustained release formulations placed in certain tissues including blood. Suitable examples of sustained release carriers include semipermeable polymer matrices in the form of shaped articles, e.g. suppositories, or microcapsules. Implantable or microcapsular sustained release matrices include polylactides (U.S. Pat. No. 3,773,919; EP 58,481) copolymers of L-glutamic acid and gamma ethyl-L-glutamate (U. Sidman et al., 1985, *Biopolymers* 22(1): 547-556), poly(2-hydroxyethyl-methacrylate) or ethylene vinyl acetate (R. Langer et al., 1981, *J. Biomed. Mater. Res.* 15: 167-277 and R. Langer, 1982, *Chem. Tech.* 12: 98-105). Liposomes containing the hybrid immunoglobulin are prepared by well-known methods: DE 3,218,121A., Epstein et al. 1985, *Proc. Natl. Acad. Sci. USA*, 82:3688-3692; Hwang et al., 1980, *Proc. Natl. Acad. Sci. USA*, 77:4030-4034; EP 52322A; EP 36676A; EP 88046A; EP 143949A; EP 142541A; Japanese patent application 83-11808; U.S. Pat. Nos. 4,485,045 and 4,544,545; and UP 102,342A. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal rate of the polypeptide leakage.

Sustained release polypeptide preparations are implanted or injected into proximity to the site of inflammation or therapy, for example adjacent to arthritic joints or peripheral lymph nodes.

The dose of the novel polypeptide administered will be dependent upon the properties of the hybrid employed, e.g. its binding activity and in vivo plasma half-life, the concentration of the hybrid in the formulation, the administration route for the hybrid, the site and rate of dosage, the clinical tolerance of the patient involved, the pathological condition afflicting the patient and the like, as is well within the skill of the physician.

The polypeptides of this invention may also be administered along with other pharmacologic agents used to treat the conditions listed above, such as antibiotics, anti-inflammatory agents, and anti-tumor agents. It may also be useful to administer the polypeptide along with other therapeutic proteins such as gamma-interferon and other immunomodulators.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The

starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

In particular, it is preferred that these plasmids have some or all of the following characteristics: (1) possess a minimal number of host-organism sequences; (2) be stable in the desired host; (3) be capable of being present in a high copy number in the desired host; (4) possess a regulatable promoter; and (5) have at least one DNA sequence coding for a selectable trait present on a portion of the plasmid separate from that where the novel DNA sequence will be inserted. Alteration of plasmids to meet the above criteria are easily performed by those of ordinary skill in the art in light of the available literature and the teachings herein. It is to be understood that additional cloning vectors may now exist or will be discovered which have the above-identified properties and are therefore suitable for use in the present invention and these vectors are also contemplated as being within the scope of this invention.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 µg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 µg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37° C. are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. et al., *Nucleic Acids Res.*, 8: 4057 (1980).

"Dephosphorylation" refers to the removal of the terminal 5' phosphates by treatment with bacterial alkaline phosphatase (BAP). This procedure prevents the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Procedures and reagents for dephosphorylation are conventional. Maniatis, T. et al., *Molecular Cloning* pp. 133-134 (1982). Reactions using BAP are carried out in 50 mM Tris at 68° C. to suppress the activity of any exonucleases which are present in the enzyme preparations. Reactions are run for 1 hour. Following the reaction the DNA fragment is gel purified.

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic

acid fragments (Maniatis, T. et al., *Id.*, p. 146). Unless otherwise provided, ligation is accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 μ g of approximately equimolar amounts of the DNA fragments to be ligated.

"Filling" or "blunting" refers to the procedures by which the single stranded end in the cohesive terminus of a restriction enzyme-cleaved nucleic acid is converted to a double strand. This eliminates the cohesive terminus and forms a blunt end. This process is a versatile tool for converting a restriction cut end that may be cohesive with the ends created by only one or a few other restriction enzymes into a terminus compatible with any blunt-cutting restriction endonuclease or other filled cohesive terminus. Typically, blunting is accomplished by incubating 2-15 μ g of the target DNA in 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM NaCl, 10 mM Tris (pH 7.5) buffer at about 37° C. in the presence of 8 units of the Klenow fragment of DNA polymerase I and 250 μ M of each of the four deoxynucleoside triphosphates. The incubation generally is terminated after 30 min. phenol and chloroform extraction and ethanol precipitation.

It is presently believed that the three-dimensional structure of the compositions of the present invention is important to their functioning as described herein. Therefore, all related structural analogs which mimic the active structure of those formed by the compositions claimed herein are specifically included within the scope of the present invention.

It is understood that the application of the teachings of the present invention to a specific problem or situation will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use, and manufacture appear below, but should not be construed to limit the invention. All literature citations herein are expressly incorporated by reference.

EXAMPLES

All references in these examples to the "Mel 14" monoclonal antibody or to "Mel 14" refer to a monoclonal antibody directed against a purported murine form of a lymphocyte surface protein, as described by Gallatin, et al., *supra*, Nature 303, 30 (1983), specifically incorporated by reference. The use of Mel 14 is no longer needed to practice this invention, however, due to the provision herein of full sequences for the DNA and amino acids of the LHR.

EXAMPLE 1.

Purification and Cloning of MLHR

Isolation of a cDNA Clone Encoding the MLHR

MLHR was isolated from detergent-treated mouse spleens by immunoaffinity chromatography using the Mel 14 monoclonal antibody.

In a typical preparation, 300 spleens from ICR female mice (16 weeks old) were minced and then homogenized with a Potter-Elvehjem tissue grinder in 180 ml of 2% Triton X-100 in Dulbecco's PBS containing 1 mM PMSF and 1% aprotinin. Lysis was continued for 30 minutes on a shaker at 4° C. The lysate was centrifuged successively at 2,000 \times G for 5 minutes and at 40,000 \times G for 30 minutes.

The supernatant was filtered through Nitex screen and then precleared by adsorption with rat serum coupled to cyanogen bromide-activated Sepharose 4B (10

ml of packed gel). The rat serum was diluted 1:10 for coupling with conjugation carried out according to the manufacturer's instructions. The flow through was applied to a 3 ml column of MEL-14 antibody coupled at 0.5 mg per ml to Sepharose 4B. All column buffers contained sodium azide at 0.02%.

The column was washed with 25 ml of 2% Triton X-100 in PBS followed by 25 ml of 10 mM CHAPS in the same buffer. Antigen was released by addition of 10 ml of 10 mM CHAPS in 100 mM glycine, 200 mM NaCl, pH 3 and neutralized by collection into 1 ml of 1M TRIS HCl, pH 7.6. After the column was washed with 20 mM triethylamine, 200 mM NaCl, pH 11 and re-equilibrated in 10 mM CHAPS in PBS, the neutralized antigen, diluted into 100 ml of the column buffer, was re-applied and the wash and release steps were repeated.

The purified protein was concentrated in a Centricon 30 (Amicon, Inc.) and analyzed by SDS-PAGE (7.5% acrylamide) with the use of silver staining for visualization. A typical purification yielded 30-40 μ g of antigen per 300 mice based upon comparisons with orosomucoid standards.

As can be seen in FIG. 4A, a polyacrylamide gel of the purified material showed a diffuse band migrating at approximately 90,000 daltons, and a higher molecular weight protein at around 180,000 daltons. The ratio of the 90,000 dalton to the 180,000 dalton component was 10:1 or greater in all of a large series of preparations. The material was visualized by silver staining of a 10% polyacrylamide gel.

Gas phase Edman degradation of the 90,000 dalton band resulted in the identification of a single N-terminal sequence (FIG. 4B), including the very N-terminal amino acid. 38 N-terminal amino acids were identified, with four gaps (X) at positions 1,19,33, and 34. The asparagine (N) at position 22 was inferred from the absence of an amino acid signal at this position combined with the following tyrosine (Y) and threonine (T) residues, resulting in an N-linked glycosylation site consensus sequence (NXT/S).

The 13-sequence residue shown in FIG. 4B above the 38 residue long N-terminus is that previously deduced by Siegelman et al., *supra*, using radioactively-labelled amino acid sequencing, which shows a high degree of homology (11 of 13 residues) with the sequence of the LHR determined here.

No ubiquitin sequence was obtained in any of the three sequencing runs that were done with two separate MLHR preparations. Conceivably, this modification was absent in the mouse splenocytes or the N-terminus of the ubiquitin is blocked to Edman degradation in the LHR from this source.

The amino acid sequences of FIG. 2 were compared with known sequences in the Dayhoff protein data base, through use of the algorithm of Lipman, D. et al., Science 227, 1435-1441 (1981).

The residues in FIG. 4B which are underlined between amino acids 7 and 15 were chosen to produce the oligonucleotide probe shown in FIG. 4C. A 32-fold redundant 26-mer oligonucleotide probe was designed from these residues and synthesized on an Applied Biosystems oligonucleotide synthesizer. All of the possible codon redundancies were included in this probe, with the exception of the proline at position 9, where the codon CCC was chosen based upon mammalian codon usage rules.

Screening of a murine spleen cDNA library obtained from dissected mouse spleens with this probe resulted in the isolation of a single hybridizing cDNA clone. Procedurally, 600,000 plaques from an oligo, dT-primed lambda gt 10 murine spleen cDNA library produced from mRNA isolated from murine splenocytes with 5 µg/ml Concanavalin A for 6 hours were plated at 50,000 phage per plate (12 plates) and hybridized with the P³² labeled 32-fold redundant 26-mer oligonucleotide probe shown in FIG. 4C, in 20% formamide, 5XSSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5× Denhardt's solution, 10% dextran sulfate, and 20 micrograms/ml denatured, sheared salmon sperm DNA overnight at 42° C. These parameters are referred to herein as "stringent conditions". The filters were washed in 1X SSC, 0.1% SDS at 42° C. for 2×30 minutes and autoradiographed at -70° C. overnight. A single duplicate positive clone was rescreened, the EcoRI insert was isolated and inserted into M13 or PUC 118/119 vectors and the nucleotide sequence determined from single stranded templates using sequence-specific primers.

FIG. 2 shows the complete DNA sequence of the 2.2 kilobase EcoRI insert contained in this bacteriophage. The longest open reading frame begins with a methionine codon at position 106-108. A Kozak box homology is found surrounding this methionine codon, suggesting that this codon probably functions in initiating protein translation. A protein sequence containing 373 amino acids of approximately 42,200 daltons molecular weight is encoded within this open reading frame. The translated protein shows a sequence from residues 40 to 76 that corresponds exactly with the N-terminal amino acid sequence determined from the isolated MLHR.

This result suggests that the mature N-terminus of the MLHR begins with the tryptophan residue at position 39. However, it is believed that some proteolytic processing of the actual N-terminus of the LHR may have occurred during the isolation of the protein.

A hydrophobicity profile of the protein reveals an N-terminally located hydrophobic domain that could function as a signal sequence for insertion into the lumen of the endoplasmic reticulum. The deduced sequence for positions 39 to 333 is predominantly hydrophilic followed by a 22 residue hydrophobic domain, which is characteristic of a stop transfer or membrane anchoring domain.

The putative intracellular region at the very C-terminus of the protein is quite short, only 17 residues in length. On the immediate C-terminal side of the predicted membrane-spanning domain are several basic amino acids, a feature typical of junctions between membrane anchors and cytoplasmic domains of cell surface receptors. Yarden et al., Nature. A single serine residue, potentially a site for phosphorylation, is present within the putative cytoplasmic domain.

The protein contains ten potential N-linked glycosylation sites, all of which are within the projected extracellular domain. The absence of asparagine at position 60 (residue 22 of the mature protein) in the peptide sequencing analysis confirms glycosylation at this site and establishes the extracellular orientation of this region. The coding region contains a total of 25 cysteine residues, although 4 of these cysteine residues are located within the putative leader sequence.

Protein Motifs Within the MLHR

As shown in FIG. 6, comparison of the deduced MLHR amino acid sequence to other proteins in the Dayhoff protein sequence databank by using the fastp program (Lipman, D., and Pearson, W., Science 227, 1435-1441, 1985) revealed a number of interesting sequence homologies.

Proteins with the highest sequence homology scores are shown with boxes surrounding the regions of greatest sequence homology. The numbers at the beginning of the sequences show the position within the proteins where these homologous sequences are located.

FIG. 6A shows that the N-terminal motif of the LHR (residues 39 to 155) has certain carbohydrate binding protein homologies, as listed (the percentage of homology of these sequences to the MLHR are given in parentheses, and the references indicated are provided after the Examples): Drickamer; the amino acid residues found by Drickamer et al. (1), MuLHR; the MLHR sequence, Hu.HepLec (27.8%); human hepatic lectin (2), Barn.Lec (25%); acorn barnacle lectin (3), Ra. HepLec (23.5%); rat hepatic lectin (4), Ch.HepLec (27.5%); chicken hepatic lectin (5), Hu.IgERec (28.6%); human IgE receptor (6), Ra.HepLec2 (22.6%); rat hepatic lectin 2 (7), Ra.ASGRec (22.6%); rat asialoglycoprotein receptor (8), Ra.IRP (25.6%); rat islet regenerating protein (9), Ra.MBP (26.1%); rat mannose binding protein (10), Ra.MBDA (26.1%); rat mannose binding protein precursor A (11), Ra.KCBP (27%); rat Kupffer cell binding protein (12), FlyLec (23.1%); flesh fly (Sarcophaga) lectin (13), and Rab.Surf (20.9%); rabbit lung surfactant (14).

As can be seen, FIG. 6A shows that the most N-terminally localized motif of the LHR shows a high degree of homology with a number of calcium-dependent animal lectins, i.e., C-type lectins (1). These include but are not limited to, various hepatic sugar binding proteins from chicken, rat, and human, soluble mannose-binding lectins, a lectin from Kupffer cells, the asialoglycoprotein receptor, a cartilage proteoglycan core protein, pulmonary surfactant apoproteins, and two invertebrate lectins from the flesh fly and acorn barnacle. Although the complement of "invariant" amino acids initially recognized by Drickamer and colleagues, supra, as common to C-type animal lectins are not completely conserved in the carbohydrate binding domain of the MLHR, the degree of homology at these residues and at other positions is apparent. The known lectins belonging to the C-type family exhibit a range of sugar-binding specificities including oligosaccharides with terminal galactose, N, acetylglucosamine, and mannose (1).

The fact that there are many residues that are found to be invariant in all of these carbohydrate binding proteins, strongly suggests that this region functions as a carbohydrate binding domain in the MLHR and apparently explains the observed ability of lymphocytes to bind to the specialized endothelium of lymphoid tissue in a sugar- and calcium-dependent manner. In some embodiments, the carbohydrate binding domain of the LHR alone, without any flanking LHR regions, is used in the practice of this invention.

The next motif (residues 160-193) that is found almost immediately after the completion of the carbohydrate binding domain shows a high degree of homology to the epidermal growth factor (egf) family. FIG. 6B shows epidermal growth factor (egf) homologies: MLHR; the MLHR sequence, Notch (38.5%); the Dro-

Drosophila melanogaster notch locus (15). *S. purp* (31.7%); *Strongylocentrotus purpuratus* egf-like protein (16). *Pro.Z* (34.1%); bovine protein Z (17). *Fact.X* (34.2%); coagulation factor X (18). *Fact.VII* (27.3%); coagulation factor VII (19). *Fact.IX* (33.3%); coagulation factor IX (20). *Lin-12* (32.1%); *Caenorhabditis elegans* *Lin-12* locus (21). *Fact. XII* (26%); coagulation factor XII (22). and *Mu.egf* (30%); murine egf (23).

As can be seen in FIG. 6B, the greatest degree of homology in this region of the MLHR is found with the *Drosophila* neurogenic locus, notch, although there is also significant homology to a number of other members of this large family. The variable location of this domain among the members of this family suggests that this region may be contained within a genomic segment that can be shuffled between different proteins for different functions.

In addition to 6 cysteine residues, virtually all members of this family share three glycine residues. The conservation of cysteine and glycine residues is consistent with the possibility of a structural role for this region in the LHR. It is believed that this domain may place the N-terminally localized carbohydrate binding region in an appropriate orientation for ligand interaction. It is further believed that this domain may serve to strengthen the interaction between the lymphocyte and endothelium by binding to an egf-receptor homologue on the endothelium surface.

The final protein motif in the extracellular region of the MLHR is encoded from amino acids 197 to 328. This region of the glycoprotein contains two direct repeats of a 62 residue sequence that contains an amino acid motif that bears a high degree of homology to a number of complement factor binding proteins (FIG. 6C).

FIG. 6C shows complement binding protein homologies: MLHR; MLHR sequence. *HuComH* (31.9%); human complement protein H precursor (24). *MuComH* (28.9%); murine complement protein H precursor (25). *HuBeta* (25.6%); human beta-2-glycoprotein 1 (26). *HuCR1* (29.9%); human CR1 (27). *EBV/3d* (25%); human Epstein-Barr virus/C3d receptor (28). *HuC2* (27.1%); human complement C2 precursor (29). *HuB* (23.1%); human complement factor B (30). *MuC4b* (22%); murine C4b-binding precursor (31). *HuCl* (29.2%); human Cls zymogen (32). *HuCAF* (26.1%); human C4b binding protein (33). *HuDAF* (27.1%); human decay accelerating factor (34). *Vac-SecP* (26.2%); vaccinia virus secretory peptide (35).

These proteins, which encode a wide range of multiples of this repeated domain, include, among others, the human and murine complement H precursors, the human beta 2 glycoprotein, the Epstein Barr virus/C3d receptor, the human C4b binding protein, the decay accelerating factor, and the vaccinia virus secretory polypeptide.

FIG. 7C shows the homologies between the two direct repeats in the MLHR and the direct repeats found in proteins contained within the complement binding family. Many of the amino acids that are conserved in this class of complement binding proteins, including a number of conserved cysteine residues, are also found in the 2 repeats in this region of the MLHR.

Interestingly, the two repeats contained within the MLHR are not only exact duplications of each other at the amino acid level, they also show exact homology at the nucleotide sequence level (nucleotide residues 685-865 and 866-1056). While it is possible that this

result is due to a cloning artifact, a duplicated region has been found in a number of other clones isolated from a separate cDNA library produced from the MLHR expressing cell line, 38C13 (available from Stanford University, Palo Alto, Calif., U.S.A.), as well as in a human homologue of the MLHR (discussed, infra.). Furthermore, a number of other genes, most notably the *Lp(a)* gene, show an even higher degree of intragenic repeat sequence conservation of this domain. These results suggest that the MLHR, like other members of the complement binding family, contains multiple repeats of this binding domain.

In conclusion, it appears that the extracellular region of the MLHR contains three separate protein motifs that have been joined together to serve a new function or functions. A summary of the protein motifs contained within this glycoprotein is shown in FIG. 7.

EXAMPLE 2

Cloning of HuLHR

Generally as described in the previous example, the 2.2 kb *EcoRI* insert of the murine Mel 14 antigen cDNA clone described above was isolated, labeled to high specific activity by randomly primed DNA polymerase synthesis with P^{32} triphosphates, and used to screen 600,000 clones from an oligo dT primed lambda gt10 cDNA library derived from human peripheral blood lymphocyte mRNA obtained from primary cells. The filters were hybridized overnight at 42° C. in 40% formamide, 5×SSC (1×SSC is 30 mM NaCl, 3 mM trisodium citrate), 50 mM sodium phosphate (pH6.8), 10% dextran sulfate, 5×Denhardt's solution and 20 micrograms/ml sheared, boiled salmon sperm DNA. They were washed 2×40 minutes in 0.2×SSC, 0.1% sodium dodecyl sulfate at 55° C. 12 clones (approximately 1 positive per plate of 50,000 phage) were picked; and the largest *EcoRI* insert (~2.2 kilobases) was isolated and the DNA sequence was determined by dideoxynucleotide sequencing in the bacteriophage m13 using sequence-specific primers.

This ~2.2 kb clone encoded an open reading frame of 372 amino acids with a molecular weight of approximately 42,200 daltons that began with a methionine which was preceded by a Kozak box homology. The encoded protein contained 26 cysteine residues and 8 potential N-linked glycosylation sites. A highly hydrophobic region at the N-terminus of the protein (residues 20-33) was a potential signal sequence, while another highly hydrophobic C-terminally located region of 22 amino acids in length (residues 335-357) was a potential stop transfer or membrane anchoring domain. This C-terminal hydrophobic region was followed by a charged, presumably cytoplasmic, region.

Comparison of the nucleotide sequence of this human clone with that previously found for the MLHR showed a high degree of overall DNA sequence homology (~83%). The relative degrees of amino acid sequence conservation between the MLHR and the HuLHR in each of the LHR domains are: carbohydrate binding domain—83%; egf-like domain—82%; complement binding repeat 1—79%; complement binding repeat 2—63%; overall complement binding domain—71%; and transmembrane domain—96%.

Comparison of the published Hermes sequence, Jalkanen, supra, with the HuLHR sequence of FIG. 1 reveals a lack of sequence homology.

EXAMPLE 3

Expression of the MLHR

In order to prove conclusively that the murine cDNA clone isolated here encoded the MLHR, the clone was inserted into an expression vector and analyzed in a transient cell transfection assay. Expression of the HuLHR was performed in a similar fashion.

The EcoRI fragment containing the open reading frame described above (the ~2.2 kilobase EcoRI fragment whose sequence is shown in FIG. 2) was isolated and ligated into the pRK5 vector which contains a cytomegalovirus promoter (Eaton, D., et al., *Biochemistry* 25, 8343-8347, 1986; U.S. Ser. No. 07/097,472), now abandoned. A plasmid containing the inserted cDNA in the correct orientation relative to the promoter was selected and transfected onto 293 human embryonic kidney cells using CaPO_4 precipitation.

After 2 days the cells were incubated with 500 microcuries each of S^{35} cysteine and methionine. Lysates and supernatants were prepared as previously described (Lasky, L., et al., *Cell* 50, 975-985, 1987) and immunoprecipitated with Mel 14 monoclonal antibody (purified by immunoaffinity chromatography) by utilizing an anti-rat IgG polyclonal antibody in a sandwich between the Mel 14 monoclonal antibody and protein A sepharose.

At the same time, the B-cell lymphoma, 38C13, a cell known to express the MLHR, were either labeled metabolically with either methionine or cysteine, for analysis of the supernatant MLHR, or the cell-surface glycoproteins were labeled with I^{125} and lactoperoxidase for analysis of cell-associated LHR and analyzed by Mel 14 antibody immunoprecipitation.

The resultant immunoprecipitates were analyzed on 7.5% polyacrylamide SDS gels and autoradiographed overnight at -70°C .

The results of these assays are shown in FIG. 5. In that figure, the lanes A-F signify the following:

- A. Lysates of 293 cells transfected with a MLHR expression plasmid immunoprecipitated with Mel 14 monoclonal antibody.
- B. Supernatants of 293 cells transfected with a MLHR expression plasmid immunoprecipitated with Mel 14 monoclonal antibody.
- C. Lysates of 293 cells transfected with a plasmid expressing the HIV gp120 envelope glycoprotein immunoprecipitated with the Mel 14 monoclonal antibody.
- D. Supernatants of 293 cells transfected with the HIV envelope expression plasmid immunoprecipitated with the Mel 14 monoclonal antibody.
- E. Supernatants of 38C13 cells immunoprecipitated with the Mel 14 monoclonal antibody.
- F. Lysates of 38C13 cells surface labeled with I^{125} and immunoprecipitated with the Mel 14 monoclonal antibody.

As can be seen in FIG. 5, cells transfected with this construct produce two cell-associated proteins that reacted specifically with the Mel 14 antibody. The cell associated proteins migrated at approximately ~70,000 daltons and ~85,000 daltons, suggesting that the ~42,200 dalton core protein becomes glycosylated in the transfected cells. The larger band was shifted in molecular weight following sialidase treatment (data not shown), suggesting that it is a relatively mature form of the glycoprotein, whereas the lower molecular

weight band was resistant to the enzyme, indicating that it may be a precursor form.

FACS analysis of transiently transfected cell lines with the Mel 14 antibody showed that a portion of the LHR expressed in these cells was detectable on the cell surface (data not shown).

The higher molecular weight glycoprotein produced in the transfected cell line was found to be slightly smaller than that produced by the Peripheral Lymph Node-homing B-cell lymphoma 38C13 (FIG. 5, lane F), a result that has been found in other transfected cell lines and may be due to cell-specific differences in glycosylation.

Interestingly, both the 38C13 cells and the transfected human cells appeared to shed a smaller molecular weight form of the MLHR into the medium (FIG. 5, lanes B and E). The nature of this shed molecule is unclear, although its reduced molecular weight suggests that it may be a cleavage product of the cell surface form resulting from proteolysis near the membrane anchor.

In conclusion, these results convincingly demonstrate that the cDNA clone that we have isolated encodes the MLHR.

EXAMPLE 4

Construction, Purification, and Analysis of Truncated MLHR-IgG Chimeras

FIG. 8 shows the construction of MLHR-IgG chimeras containing the lectin, lectin-egf, and lectin-egf-complement regulatory motifs. The top of the figure illustrates the protein domains of the murine lymphocyte homing receptor (MLHR) including the N-terminal signal sequence (SS), the lectin, epidermal growth factor (egf), and duplicated complement regulatory domains (CDB) as well as a transmembrane anchor domain (TMD) and a short cytoplasmic sequence. The three truncated MLHR-IgG chimeras which contain the lectin (MLHR-L + IgG), the lectin and egf (MLHR-LE + IgG) and the lectin, egf, and two complement regulatory motifs (MLHR-LEC + IgG) are also shown in FIG. 8. These truncated proteins are all joined to a human heavy chain gamma 1 region just upstream of the hinge domain (H) such that these chimeras contain the two cysteine residues (C) of the hinge responsible for immunoglobulin dimerization as well as the CH2 and CH3 constant regions. A previously characterized human heavy chain IgG 1 constant region cassette (Capon et al., supra 1989) was utilized. Junctional sites between the LHR and human IgG sequences was chosen such that the joining of the molecules near the hinge region resulted in chimeric molecules that were efficiently synthesized and dimerized in the absence of any light chain production. In addition, the use of the human IgG 1 constant region obviates any difficulties due to cross reactivity with endogenous murine IgGs in the immunohistochemical experiments described below.

As can be seen from FIG. 9, these chimeras were efficiently synthesized and secreted in these transient transfection assays. The reactivity of these chimeras with protein A sepharose in the absence of added antibodies demonstrates that the constant region domains are normally folded. FIG. 9 illustrates that these molecules dimerize under non-reducing conditions, demonstrating that the hinge region is fully functional in these chimeras. Finally, the protein A reactivity also allows

for the purification of these chimeras to near homogeneity on protein A sepharose columns. The results herein demonstrate the production of antibody-like entities whose "variable" domain may be said to be derived from the MLHR while the constant domain is derived from the human IgG gamma 1 heavy chain.

CONSTRUCTION OF CHIMERAS

Starting with a previously described MLHR-PRK5 expression plasmid (Eaton et al., 1986.; Lasky et al., *Cell* 50:975-985, 1987) and a cDNA copy of a human heavy chain IgG (Capon et al., *Nature* 337:525-531, 1989), an 1100 bp HindIII fragment encoding the CH1-CH3 regions of the human IgG 1 constant region was inserted 3 prime of the polyA site of the MLHR cDNA. This plasmid was converted to single stranded template by utilizing an m13 origin of replication and the K07 helper phage, after which regions between the hinge and the lectin, egf, and second complement binding repeat N-terminal to the putative trans membrane anchoring region) were looped out with 48-mer oligonucleotides by in vitro mutagenesis (Zoller and Smith, 1982). The resultant mutants were screened with 32P-labeled 21-mer oligonucleotides spanning the deletion junctions, and the isolated mutants were sequenced using super-coil sequencing.

Correct mutants were tested for expression by transfection onto human kidney 293 cells using previously described methods. 35S methionine and cysteine labeled supernatants were analyzed by immunoprecipitation with protein A sepharose beads in the absence of added antibodies. The precipitated proteins were analyzed on 7.5% polyacrylamide-SDS gels either with or without reduction with beta mercaptoethanol. Plasmids that resulted in correctly expressed chimeras were introduced into 293 cells by transfection in the presence of selective plasmids encoding resistance to G418 as well as dihydrofolate reductase. Clones were selected in G418, and the incorporated plasmids were amplified in the presence of methotrexate. Permanent cell lines expressing high levels of each construct were grown to large scale in T-flasks, and the cell supernatants were clarified by centrifugation and filtration. The resultant supernatants were concentrated by Amicon filtration and passed over standard protein A-sepharose columns, washed with PBS, and eluted with 0.1M Acetic Acid, 0.15M NaCl (pH 3.5). The eluted material was immediately neutralized with 3M Tris, pH 9, and quantitated by SDS gel electrophoresis as well as an ELISA assay.

The gel electrophoresis results described in the preceding paragraph are shown in FIG. 9. Reduced proteins are shown in lanes A-F., non-reduced proteins in lanes G-I, and purified proteins in lanes J-L. Molecular weights of markers are shown in kilodaltons. Lane identifications are as follows: A. Secreted MLHRLEC-IgG, B. Intracellular MLHRLEC-IgG, C. Secreted MLHRLE-IgG, D. Intracellular MLHRLE-IgG, E. Secreted MLHRL-IgG, F. Intracellular MLHRL-IgG., G. Secreted MLHRLEC-IgG, H. Secreted MLHRLE-IgG, I. Secreted MLHRL-IgG, J. Purified MLHRLEC-IgG, K. Purified MLHRLE-IgG, and L. Purified MLHRL-IgG.

Isolated LHR-IgG chimeras were quantitated using an ELISA format that consisted of an anti-human IgG 1-specific mouse monoclonal antibody coating microtitre wells. Unknown samples as well as highly purified human CD4-IgG 1 immunoadhesin standard were incubated with antibody-coated plates, after which the

plates were washed, and the bound material was reacted with horse radish peroxidase-conjugated goat-anti human IgG 1, followed by further washes and addition of substrate. This quantitative assay allowed for the measurement of sub-nanogram quantities of isolated LHR-IgG chimeras.

Analysis of MLHR-IgG Chimera PPME Reactivity by ELISA

The ability of various IgG chimeras to recognize the yeast cell wall carbohydrate, polyphosphomannan ester or PPME, was analyzed in an ELISA format as previously described (Imai et al., 1989). Briefly, approximately equivalent amounts of the purified chimeras were coated onto microtitre wells overnight at 4° C. Non-specific sites were blocked with BSA, after which the bound antigens were reacted with a 5 microgram per ml solution of PPME. Bound carbohydrate was detected with a polyclonal antibody directed against it and standard (Vector) immunohistochemical staining reagents. Inhibition with Mel 14 was performed by pre-incubating MLHRLEC-IgG containing wells with the monoclonal antibody before the addition of PPME, while the calcium dependence of the homing receptor-carbohydrate interaction was demonstrated by inclusion of 10 mM EGTA during the binding reaction. Various other additives were added before PPME incubation in assays examining inhibition. After 1 hr at 22° C., the plates were washed and incubated with a rabbit polyclonal antibody directed against PPME for 1 hr at 22° C. Plates were washed and incubated with Vector ABC-AP for 30 minutes, washed, and developed. The resulting assays were measured on a plate reader. Carbohydrates used in the inhibitory assays were obtained from Sigma Chemical Co. (St. Louis, Mo.)

Results of the PPME binding analysis are shown in FIG. 10. The lanes contain the following MLHR-IgG chimeras: A. Binding of PPME to MLHRL-, MLHRLE- and MLHRLEC-IgG chimeras. B. Inhibition of MLHRLEC-IgG-PPME binding with Mel 14 monoclonal antibody and EGTA. C. Inhibition of MLHRLEC-IgG-PPME binding with other carbohydrates.

Previous work had demonstrated that the LHR was able to bind to a yeast cell wall mannan, polyphosphomannan ester or PPME (Yednock et al., *J. Cell Biol.* 104:725-731, 1987), and that this binding inhibited the ability of lymphocytes to adhere to peripheral lymph node high endothelial vesicles, in agreement with the supposition that the peripheral lymph node LHR lectin domain may recognize a carbohydrate on the peripheral lymph node endothelium. In addition, the MEL 14 antibody was found to inhibit the binding of PPME to the lymphocyte surface (Yednock et al., supra 1987), consistent with the notion that this carbohydrate bound within the lectin domain of the peripheral lymph node LHR.

The chimera that contained the lectin, egf, and duplicated complement binding repeat structures was found to bind PPME. This binding was inhibitable by the Mel 14 antibody, in agreement with data demonstrating that the MLHRLEC-IgG chimera was recognized by this antibody (data not shown), and was quantitatively comparable to that found previously using MLHR isolated from spleen cells (Imai et al., submitted for publication, 1989), suggesting that it represented the same protein-carbohydrate interaction that has been found with the LHR on the lymphocyte surface (Yednock et al. supra

1987). In addition, the binding was also found to be calcium dependent (Stoolman and Rosen, *J. Cell Biol* 96:722-729, 1983), implying that the type C or calcium-dependent lectin domain (Drickamer, *J. Biol. Chem.* 263:9557-9560, 1988) was at least partly responsible for this interaction, as has been shown for the lymphocyte-associated receptor (FIG. 9b).

Previous work demonstrated that a variety of carbohydrates besides PPME were capable of being recognized by the spleen derived MLHR (Yednock et al., supra 1987; Imai et al., supra 1989). These included fucoidin, dextran sulfate, and brain derived sulfatides. The ability of these carbohydrates to inhibit the interaction between the MLHRLEC-IgG chimera and PPME was examined to investigate the specificity of this molecule versus the previously described spleen-derived glycoprotein (Imai et al., supra 1989). As can be seen from FIG. 9, fucoidin dextran sulfate, and sulfatide are all able to inhibit the interactions between PPME and MLHRLEC-IgG, implying that the carbohydrate specificity of this recombinant-derived protein mimics that previously described for the naturally occurring protein. The lack of inhibition by two other charged carbohydrates, chondroitin sulfate and heparin, suggests that the inhibition is due to specific carbohydrate recognition and not to non-specific interference due to the highly charged nature of the compounds.

Cell Blocking Assays with MLHR-IgG Chimeras

The Stampfer-Woodruff cell blocking assay (Stampfer and Woodruff, *J. Exp. Med.* 144:828-833, 1976) was performed with cryostat-cut sections of mouse peripheral lymph nodes as well as with Peyer's patch as previously described (Geoffrey and Rosen, *J. Cell Biol.* in press, 1989). Briefly, the frozen tissue sections were incubated with mesenteric lymphocytes in the presence of either the MLHR-IgG chimeras, isolated spleen-derived MLHR, or buffer alone. MLHR-IgG chimeras were included at concentrations as high as 10 micrograms per section and were pre-incubated on frozen sections before the addition of 1×10^7 cells per ml. The slides were washed, and lymphocyte attachment was measured by digital morphometry as the number of lymphocytes bound to HEV in these lymphoid organs per unit area.

In data not shown, the MLHRLEC-IgG chimera was found to inhibit the binding of lymphocytes to peripheral lymph node HEV at a level of approximately 75% inhibition while, in this assay, the spleen-derived MLHR blocked at a level of about 50%. This inhibition was calcium dependent and was blocked by the inclusion of the MEL 14 monoclonal antibody (data not shown).

Immunohistochemical Analysis of MLHR-IgG Chimeras

Isolated MLHR-IgG chimeras were utilized for immunohistochemical experiments using procedures identical to those used for monoclonal antibodies. 8-10 micron tissue sections were cut in a cryostat and fixed with 0.1 M cacodylate, 1% paraformaldehyde for 30 minutes at 4° C. The sections were washed in Dulbecco's PBS and stained with varying amounts of MLHR-IgG chimera in 5% normal mouse serum at 4° C. for 30 minutes. The sections were then washed and incubated with a second stage containing biotinylated goat anti-human Fc specific antibody (Vector). Endogenous peroxidase was eliminated by treating the sections with hydrogen

peroxide-methanol after the addition of the second stage reagent and before the addition of the Vector ABC complex. Sections were washed and incubated with substrate (AEC) for 5-10 minutes. Finally, the sections were counter-stained with aqueous hematoxylin (Biomedica) and viewed with a Zeiss Axioplan.

These immunohistochemical analyses of the three MLHR-IgG chimeras used peripheral lymph node as a tissue source. The choice of peripheral lymph node as a histology source was dictated by the large body of previous literature which demonstrated that lymphocytes bind to the HEV of this lymphoid tissue in a manner which can be blocked by MEL-14, implying that the highest level of ligand recognized by the MLHR should be in this tissue (Gallatin et al., *Nature* 304:30-34, 1983). The MLHRLEC-IgG chimera was able to stain peripheral lymph node HEV. The staining was found exclusively over the high walled endothelial cells, with no staining of ad- or abluminal regions. In addition, this staining could be blocked by the MEL 14 antibody and was dependent upon the presence of calcium, suggesting that the binding of MLHRLEC-IgG to peripheral lymph node HEV mimicked the adhesion between lymphocytes and the HEV. In concordance with the PPME binding data, the staining of peripheral lymph node HEV by MLHRLEC-IgG was inhibitable by fucoidin and dextran sulfate (FIG. 5), while chondroitin sulfate and simple mannans were incapable of inhibiting the staining reaction (data not shown), again implying that the staining reaction was due to the recognition of a carbohydrate ligand expressed on the peripheral lymph node HEV. These data reveal that this type of immunohistochemical reagent may be utilized to investigate the tissue distribution of the endothelial molecule(s) which are capable of interacting with the peripheral lymph node LHR.

The MLHR Ligand is Found in Peyer's Patches

We have found, in results of immunohistochemical assays not shown, that the MLHRLEC-IgG chimera is, surprisingly, able to recognize the endothelium of Peyer's patches specifically. The chimera appears to stain the high walled endothelium of Peyer's patches vessels containing lymphocytes. This staining is inhibitable by the MEL 14 antibody and is also calcium dependent. Interestingly, the staining of the Peyer's patches HEV appears somewhat weaker relative to that found for the staining of the peripheral lymph node HEV, implying that a lower level of the MLHR ligand(s) may be expressed in this lymphoid organ. These results demonstrate that, while other adhesion systems may be involved in this organ (Holzman et al., *Cell* 56:37-46, 1989), the ligand(s) for the peripheral lymph node LHR is expressed and, therefore, is involved in lymphocyte binding to the endothelium of this lymphoid organ.

EXAMPLE 5

Construction of CD4-IgG-MLHR-IgG Chimeras

Two previously constructed PRK plasmids were used to direct expression of MLHR-IgG and human CD4-IgG. The MLHR plasmid is as described in the previous example. The CD4-Ig plasmid is that described in Capon et al. supra, modified by the deletion of the coding region for the C_H1 domain and a portion of the hinge region up to the first cysteine residue. These plasmids were cotransfected by the standard calcium-phosphate method as described above into

human 293 cells, either together with PSV^T antigen to generate cells transiently expressing the two genes at high levels, or together with PSV^{neo}, to confer neomycin resistance for selection of cell clones stably expressing the two genes. Expression was analyzed by radioimmunoprecipitation: because CD4-IgG, LHR-IgG and CD4-IgG-LHR-IgG all contain an IgG Fc portion, they can all be precipitated directly by protein A by standard methods. Three types of molecules were detected: CD4-IgG homodimers, LHR-IgG homodimers, and CD4-IgG-LHR-IgG heterodimers. These molecules are separated to their monomeric constituents by reduction, indicating that the members of each dimer, including heterodimers, are covalently attached to one another by disulfide bonds.

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We claim:

1. Nucleic acid encoding a polypeptide fusion comprising a ligand binding partner protein containing more than one polypeptide chain, wherein the ligand binding partner protein is not a platelet growth factor receptor or an insulin receptor, one of said chains being fused to an immunoglobulin constant region through C- or N-terminal amino or carboxyl groups.
2. Nucleic acid encoding a polypeptide fusion of a ligand binding partner protein and an immunoglobulin chain, wherein the ligand binding partner protein is not a platelet growth factor receptor or an insulin receptor, said ligand binding partner protein and said immunoglobulin chain being fused through C- or N-terminal amino or carboxyl groups, and said fusion further comprising an additional fusion of an agent selected from the group consisting of a multiple subunit (chain) polypeptide, a portion of an immunoglobulin superfamily member, a toxin and a polypeptide therapeutic agent not otherwise associated with an immunoglobulin, and an immunoglobulin chain.
3. The nucleic acid of claim 2 wherein said ligand binding partner is LHR and said agent in said additional fusion is CD4.
4. Nucleic acid encoding a polypeptide fusion comprising a ligand binding partner protein which comprises a LHR and an immunoglobulin chain, in which the ligand binding partner protein and immunoglobulin are fused through C- or N-terminal amino or carboxyl groups.
5. The nucleic acid of claim 4 wherein said immunoglobulin chain is obtained from IgG1, IgG2, IgG3, IgG4, IgE, IgD or IgM.
6. The nucleic acid of claim 4 wherein in said LHR the transmembrane and the cytoplasmic domains have been deleted.
7. The nucleic acid of claim 6 wherein the transmembrane and cytoplasmic domain deleted LHR is fused at its C-terminus to the N-terminus of an human IgG immunoglobulin heavy chain constant region sequence.
8. The nucleic acid of claim 7 wherein the transmembrane and cytoplasmic domain deleted LHR is fused at its C-terminus to the N-terminus of an IgG immunoglobulin sequence beginning in the hinge region just upstream of the papain cleavage site at residue 216, taking the first residue of the heavy chain constant region to be 114.

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ARTICLES

Designing CD4 immunoadhesins for AIDS therapy

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A newly-constructed antibody-like molecule containing the gp120-binding domain of the receptor for human immunodeficiency virus blocks HIV-1 infection of T cells and monocytes. Its long plasma half-life, other antibody-like properties, and potential to block all HIV isolates, make it a good candidate for therapeutic use.

DESPITE the exquisite ability of the immune system to distinguish between self and non-self, and to put forth an impressive diversity in its antigen-recognizing repertoire, it can still be outflanked by a rapidly changing pathogen. Human immunodeficiency virus type 1 (HIV-1) is an example of such a pathogen, and, as a result, its consequences are devastating. Every individual infected with the virus is expected to develop a serious or life-threatening illness¹; no protective state has been shown to be generated in natural infections. It has not yet been possible to generate a protective response by immunizing chimpanzees with gp120, the HIV-1 envelope glycoprotein^{2,3}, or to confer passive immunity to chimpanzees using human IgG⁴. Even neutralizing antibodies made in experimental animals can block the infectivity of only a few HIV-1 isolates^{3,5}. Thus, the prospects for eliciting protective immunity against HIV-1, or for using antibodies as therapeutic agents to control HIV-1 disease are bleak. Anti-retroviral chemotherapy using dideoxynucleosides such as AZT does help some patients, but the toxicity is such that new strategies are needed⁶.

We have therefore attempted to block HIV-1 infectivity with soluble derivatives of CD4, the receptor for HIV-1, with the rationale that the CD4-binding domain of gp120 is the only part of gp120 that the virus cannot afford to change⁷. CD4 is a cell-surface glycoprotein found mostly on a subset of mature peripheral T cells that recognize antigens presented by class II MHC molecules^{8,9}. Antibodies to CD4 block HIV-1 infection of T cells^{10,11} and human cells not susceptible to HIV-1 infection become so after transfection with a CD4 cDNA¹². Gp120 binds CD4 with high affinity ($K_D \sim 10^{-9}$ M), suggesting that it is this interaction which is crucial to the entry of virus into cells^{7,13}. Indeed, we⁷ and others¹⁴⁻¹⁸ have shown that soluble rCD4, lacking the transmembrane and cytoplasmic sequences of CD4, can block HIV-1 infectivity, syncytium formation, and cell killing by gp120 (ref. 19). rCD4 blocks the infectivity of diverse HIV-1 isolates (R.B., J.G., H.M. and S.B., unpublished results),

and in theory should block all. At best, however, soluble rCD4 offers only a passive defence against the virus.

Active immunity requires a molecule such as an antibody, which can specifically recognize a foreign antigen or pathogen and mobilize a defence mechanism. Antibodies comprise two functionally independent parts, a rather variable domain (Fab), which binds antigen, and an essentially constant domain (Fc), providing the link to effector functions such as complement or phagocytic cells. It is almost certainly the lack of an antigen-binding domain which can neutralize all varieties of virus that hampers the development of humoral immunity to HIV-1. We reasoned that the characteristics of CD4 would make it ideal as the binding site of an antibody against HIV-1. Such an antibody would bind and block all HIV-1 isolates, and no mutation the virus could make, without losing its capacity to infect CD4⁺ cells specifically, would evade it. We therefore set out to construct such an antibody by fusing CD4 sequences to antibody domains.

We had two major aims for our hybrid molecules; first, as pharmacokinetic studies in several species predict that the half-life of soluble CD4 will be short in humans (30-120 min; J.M., unpublished results) we wished to construct a molecule with a longer half-life; second, we wanted to incorporate functions such as Fc receptor binding, protein A binding, complement fixation and placental transfer, all of which reside in the Fc portion of IgG. The Fc portion of immunoglobulin has a long plasma half-life, like the whole molecule, whereas that of Fab is short, and we therefore expected to be able to fuse our short-lived CD4 molecule to Fc and generate a longer-lived CD4 analogue. Because CD4 is itself part of the immunoglobulin gene superfamily, we expected that it would probably fold in a way that is compatible with the folding of Fc. We have therefore produced a number of CD4-immunoglobulin hybrid molecules, using both the light and the heavy chains of immunoglobulin, and investigated their properties. We have named one

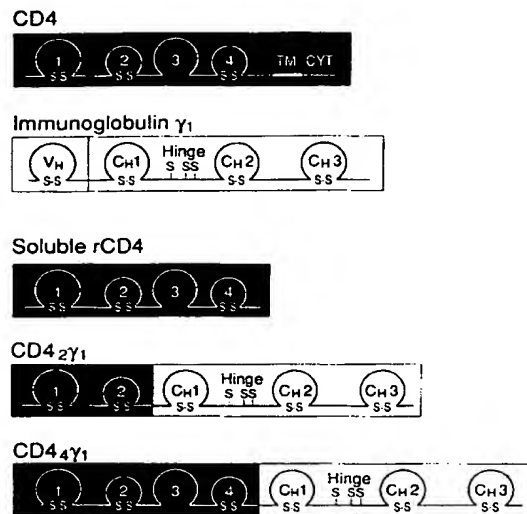


Fig. 1 Structure of cell surface CD4, human IgG1 (γ_1), soluble rCD4, and CD4 immunoadhesins (2 γ_1 and 4 γ_1). The immunoglobulin-like domains of CD4 are numbered 1 to 4; TM and CYT refer to the transmembrane and cytoplasmic domains. Soluble rCD4 is truncated after proline 368 of the mature CD4 polypeptide. This results in a secreted, soluble polypeptide with an affinity for gp120 similar to that of cell surface CD4 (ref. 7). The vertical division within IgG1 indicates the junction of the variable (VH) and constant (CH1, hinge, CH2, and CH3) regions. Disulphide bonds formed within IgG1 domains and the immunoglobulin-like domains of CD4 are indicated by (S-S). The positions of cysteine residues that form intermolecular disulphide bridges connecting the IgG1 heavy-chain hinge to light and heavy chains are indicated by (S). CD4-derived and IgG1-derived domains of 2 γ_1 and 4 γ_1 are indicated by shaded and unshaded regions, respectively. The 2 γ_1 and 4 γ_1 immunoadhesins consist of residues 1 to 180 and residues 1 to 366 of the mature CD4 polypeptide, respectively, fused to the first residue (serine 114) of the human IgG1 heavy-chain constant region.

Methods. For the expression of CD4 immunoadhesins, the sequences of CD4 and human IgG1 were fused by oligonucleotide-directed deletional mutagenesis after their insertion into a mammalian expression vector used for soluble rCD4 expression⁷. A human IgG1 heavy-chain cDNA, obtained from a human spleen cDNA library using probes based on the published sequence⁴⁷, was inserted at a unique *Xba*I site found immediately 3' of the CD4 coding region in the same reading orientation as CD4. Synthetic 48-mer oligodeoxynucleotides, complementary to the 24 nucleotides at the borders of the desired CD4 and IgG1 fusion sites, were used as primers in the mutagenesis reactions using the plasmid described above as the template⁴⁸.

particularly interesting class of these CD4-immunoglobulin hybrids 'immunoadhesins', because they contain part of an adhesive molecule²⁰ linked to the immunoglobulin Fc effector domain.

Synthesis of CD4 immunoadhesins

CD4 is an integral membrane protein with an extracellular region comprising four domains with homology to immunoglobulin variable domains^{21,22} (Fig. 1). Soluble CD4 derivatives consisting of this extracellular region bind gp120 with the same affinity as cell-surface CD4 (ref. 7). CD4 variants containing only domains 1 and 2 also bind gp120^{17,18}, but the affinity of this interaction is not known. We constructed a series of hybrid molecules consisting of the first two or all four immunoglobulin-like domains of CD4 fused to the constant region of antibody heavy and light chains (Fig. 1).

We investigated the synthesis and secretion of these hybrids using transient expression in a human embryonic kidney-derived cell line. As shown in Fig. 2, immunoglobulin light and heavy

chains are efficiently expressed in these cells, and light chain is efficiently secreted, but heavy chain is not unless a light chain is coexpressed. Thus the rules governing immunoglobulin chain secretion in these cells are the same as those for plasma or other lymphoid cells²³. We first constructed hybrids that fused CD4 with the constant regions of murine κ - or γ_1 -chains. These hybrids contained either the first two or all four immunoglobulin-like domains of CD4, linked at a position chosen to mimic the spacing between disulphide-linked cysteines seen in immunoglobulins (Fig. 1). As expected, the CD4- κ hybrids were secreted well, whereas hybrids between CD4 and mouse γ_1 -chain were expressed but not secreted unless a κ -chain or a CD4- κ hybrid was present.

A different and unexpected picture emerged when analogous CD4-heavy-chain hybrids were constructed using the constant region of human IgG1 heavy chain instead of mouse heavy chain. Such hybrids, containing either the first two or all four immunoglobulin-like domains of CD4 (named 2 γ_1 and 4 γ_1 respectively), were secreted in the absence of wild-type or hybrid light chains (Fig. 2a). Both 2 γ_1 and 4 γ_1 could be directly immunoprecipitated using *Staphylococcus aureus* protein A, which binds the Fc portion of IgG1, indicating that the protein A-binding sites of these constructs are fully functional. Indeed, both molecules can be purified to near homogeneity on protein A columns (Fig. 2b).

Structure of CD4 immunoadhesins

We examined the subunit structure of these immunoadhesin molecules using SDS-polyacrylamide gels (Fig. 2b). Without any reducing agent, the apparent relative molecular mass (M_r) of each construct doubled, demonstrating that both immunoadhesins are disulphide-linked dimers. The hinge region of each immunoadhesin contains three cysteine residues, one normally involved in disulphide bonding to light chain, the other two in the intermolecular disulphide bonds between the two heavy chains in IgG. As the molecules are dimers at least one, and perhaps all three, of these cysteine residues are involved in intermolecular disulphide bonds. We examined the capacity of 2 γ_1 and 4 γ_1 to form disulphide links with light chains. When an immunoadhesin construct was cotransfected with a light chain, the light chain produced could be precipitated by protein A. Mutagenic substitution of the first hinge-region cysteine with alanine abolished light-chain bonding, but did not affect dimerization (data not shown), indicating that this cysteine bonds the light chain in these hybrids, as in normal IgG. Thus the disulphide bond structure of these immunoadhesins seems to be analogous to that of immunoglobulins.

gp120 binding

To determine whether our immunoadhesins retain the ability to bind gp120 with high affinity, and whether the first two immunoglobulin-like domains are sufficient, we carried out saturation binding analyses with radioiodinated gp120. Binding is saturable, showing a simple mass action curve (Fig. 3a). The dissociation constant (K_d) for the interaction of each immunoadhesin with gp120, calculated by Scatchard analysis (Fig. 3a, inset), was indistinguishable from that of soluble rCD4 ($\sim 10^{-9}$ M) (Table 1). Thus, the N-terminal 170 amino acids of CD4 are sufficient for high-affinity binding. As these immunoadhesins are homodimeric, they should each have two gp120-binding sites. We examined this possibility by coating plastic microtitre wells with gp120, then adding soluble CD4 or immunoadhesins. Both immunoadhesins could bind added labelled gp120, whereas soluble rCD4, with only one gp120 binding site, could not (J. Porter and S. C., unpublished results). To confirm the bivalent nature of 2 γ_1 and 4 γ_1 , we examined their ability to agglutinate sheep red blood cells coated with gp120. Again, both CD4 immunoadhesins, but not soluble rCD4, agglutinated the cells, showing that binding to gp120 molecules on different cells is not sterically hindered.

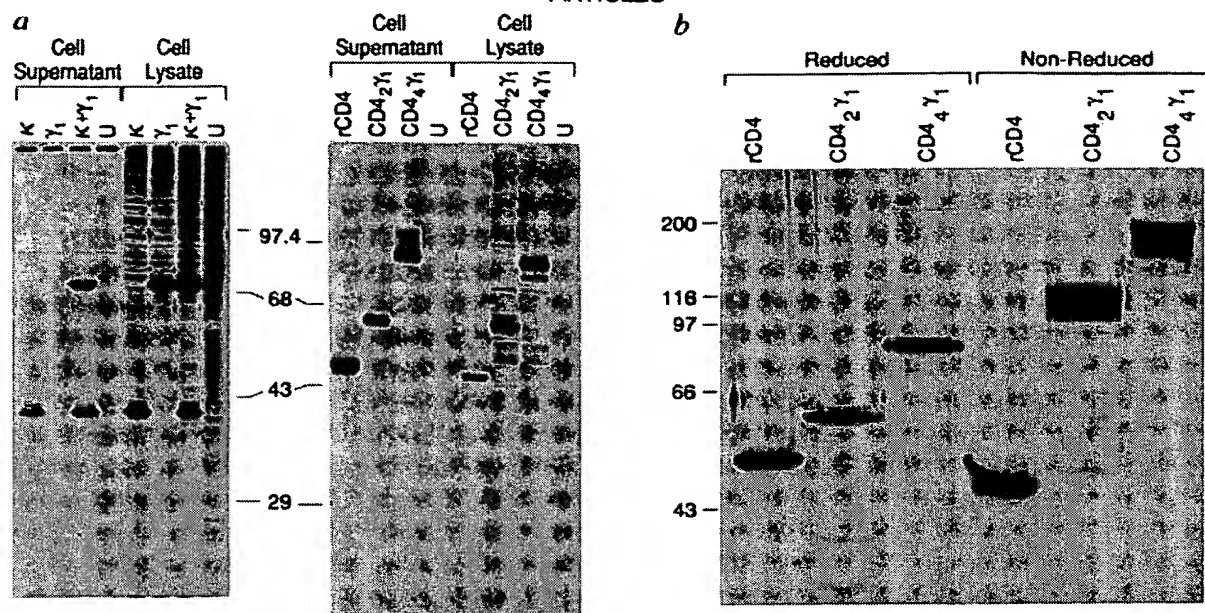


Fig. 2 Expression, secretion and subunit structure of CD4 immunoadhesins and soluble rCD4. *a*, Expression and secretion of mouse immunoglobulins, soluble rCD4 and CD4 immunoadhesins expressed in mammalian cells. Cells were transfected with vectors directing the expression of murine κ -light chain (lanes κ) or γ_1 -heavy chain (lanes γ_1) individually or together (lanes $\kappa + \gamma_1$), vectors encoding soluble rCD4 (lanes rCD4), and the CD4 immunoadhesins 2 γ_1 (lanes CD4 γ_1) or 4 γ_1 (lanes CD4 γ_2). After metabolic labelling with [35 S]methionine, cell supernatants and cell lysates were analysed by immunoprecipitation. Lanes U, untransfected cells. *b*, Subunit structure of secreted CD4 immunoadhesins and soluble rCD4. Soluble rCD4, 2 γ_1 and 4 γ_1 were purified from culture supernatants of transfected cells and analysed by electrophoresis on a 7.5% SDS-polyacrylamide gel. Samples were prepared in buffer with 10 mM dithiothreitol (DTT) (reducing conditions) or without DTT (non-reducing conditions). The positions of relative molecular mass standards are indicated (in thousands). Both immunoadhesins behaved as disulphide-linked dimers; in contrast, soluble rCD4 which is monomeric, displayed only a minor change in mobility upon reduction of its intra-molecular disulphide bonds.

Methods. *a*, Cells were transfected by a modification of the calcium phosphate procedure, labelled with [35 S]methionine, and cell lysates prepared as described⁶. Immunoprecipitation analysis was carried out as previously described⁷, with the exception that no preadsorption with Pansorbin (Calbiochem) was done, and the precipitating antibodies used were 2 μ l of rabbit anti-mouse IgG serum (Cappel) for mouse IgG heavy and light chains, 0.25 μ g of OKT4A (Ortho) for soluble rCD4, and no added antibody (Pansorbin only) for the CD4 immunoadhesins. Immunoprecipitated proteins were resolved on 10% SDS-PAGE gels, and visualized by autoradiography. *b*, CD4 immunoadhesins were purified from transfected cell supernatants by protein A affinity chromatography followed by ammonium sulphate precipitation. Purified proteins were subjected to SDS-PAGE under both reducing and non-reducing conditions and visualized by silver staining.

In vivo plasma half-life

We examined whether the immunoadhesins share the long *in vivo* half-life of antibodies. Studies of rCD4 in rabbits provide clearance data that extrapolate well to other species, including humans (J.M., unpublished results). The change in plasma concentration with time for each of the three CD4 analogues in rabbits is shown in Fig. 4. Analysis of these data reveals that soluble rCD4 has a terminal half-life in rabbits of ~15 min, whereas 4 γ_1 and 2 γ_1 have terminal half-lives of ~7 and 48 h, respectively (Table 1). Thus the half-life of 2 γ_1 in rabbits is nearly 200 times longer than that of rCD4 and comparable to that of human IgG in rabbits (4.7 days)²⁴. The half-life of 2 γ_1 in humans is expected to be longer than that in rabbits, because of the decreased proportional blood flow to eliminating organs

as species increase in size²⁵, and should be comparable with that of human IgG1 (21 days).

Our results confirm our initial hypothesis that, as in the case of immunoglobulin itself, one can increase the stability of a rapidly cleared molecule (Fab or rCD4) by fusing it to a long-lived molecule, Fc. The swift clearance of rCD4 is probably largely due to its size, M_r 55,000, which means it is just small enough to be cleared efficiently by renal filtration. One component in the increased half-lives of these molecules is therefore probably their larger size; but this cannot be the whole story as 4 γ_1 , although larger than 2 γ_1 , has a shorter half-life. Both 4 γ_1 and rCD4, but not 2 γ_1 , contain two CD4-derived Asn-linked carbohydrate sites which are glycosylated in rCD4 (R. Harris and M. Spellman, unpublished results); these sugar moieties

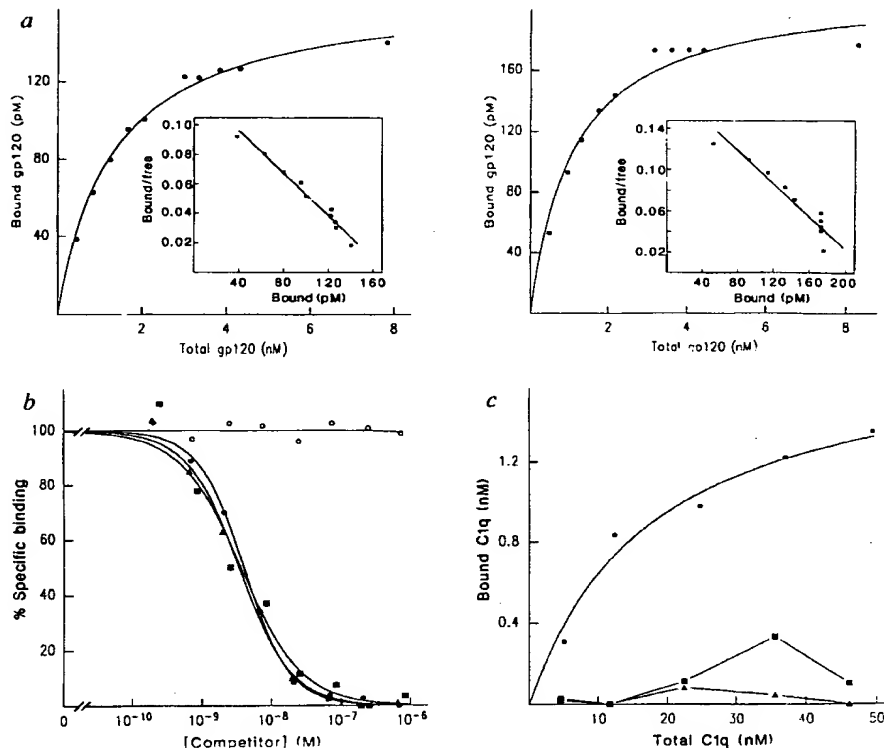
Table 1 Properties of CD4 immunoadhesins and soluble rCD4

	Calculated M_r	Subunit structure	gp120 binding (nM)*	Blocks infectivity T cells MØ	Plasma half-life in rabbits (hours)†	Fc binding (nM)*	Complement binding	Protein A binding
rCD4	41,000	monomer	2.3 ± 0.4	Yes	0.25 ± 0.01	—	No	No
4 γ_1	154,000	dimer	1.2 ± 0.1	Yes	6.7 ± 1.1	2.3 ± 0.7	No	Yes
2 γ_1	112,000	dimer	1.4 ± 0.1	Yes	48.0 ± 8.6	2.6 ± 0.3	No	Yes
IgG1	146,000	tetramer (H ₂ L ₂)	—	—	113‡	3.2 ± 0.2	Yes	Yes

* Standard error of the mean was determined using the Inplot and Scatplot programs (see Fig. 3 legend). † Standard deviation indicated in hours.

‡ Determined in ref. 24 (IgG1 has a half-life of 21 days in humans).

Fig. 3 Binding properties of CD4 immuno-adhesins. **a**, Gp120 saturation binding analysis of CD4 immuno-adhesins. Immuno-adhesin proteins 2y1 (left) or 4y1 (right) in transfected cell supernatants were incubated with increasing concentrations of purified soluble rgp120 (ref. 50) radioiodinated with lactoperoxidase. The lines drawn for the binding curves and for the Scatchard plots of the data (shown in the insets) represent the best fit as determined by unweighted least-squares linear regression analysis. Dissociation constants calculated from these results and from binding studies of gp120 to soluble rCD4 performed in parallel are given in Table 1. **b**, Binding of CD4 immuno-adhesins to Fc γ receptors on U937 cells. Competition binding analysis was carried out by mixing $0.1 \mu\text{g ml}^{-1}$ of ^{125}I -labelled human IgG1 (Calbiochem) with increasing concentrations of purified human IgG1 (solid circle), 2y1 (solid square), 4y1 (solid triangle), or soluble rCD4 (open circle) proteins. Curves drawn represent the best fit as determined by unweighted least-squares nonlinear (IgG1, 2y1 and 4y1) or linear (rCD4) regression analysis. Dissociation constants calculated from these results are shown in Table 1. **c**, C1q saturation binding analysis of CD4 immuno-adhesins. Purified anti-gp120 IgG2a mouse monoclonal antibody (solid circle), 2y1 (solid square), or 4y1 (solid triangle) proteins were aggregated by binding to gp120-coupled Sepharose, and incubated with increasing concentrations of purified human C1q (Calbiochem) radioiodinated with lactoperoxidase. The curve drawn for the anti-gp120 monoclonal antibody (mAb) represents the best fit as determined by least-squares nonlinear regression analysis; the dissociation constant for C1q binding to this gp120-aggregated anti-gp120 mAb was $\sim 1.8 \times 10^{-8}$ M.



Methods. **a**, Gp120 saturation binding analysis was carried out as described⁷ except that gp120-CD4 immuno-adhesin complexes were collected directly onto Pansorbin: binding was comparable to that observed when complexes were collected with OKT4A as for soluble rCD4. Specifically bound ^{125}I -labelled gp120 was determined from the difference in binding in the presence or absence of a 1,000-fold excess of unlabelled rgp120 and is plotted against the total ^{125}I -labelled gp120 concentration. **b**, FcR binding analysis was done essentially as described²⁷ except that after centrifugation free IgG1 was removed by aspiration of the aqueous and oil layers. Mixtures of ^{125}I -labelled human IgG1 and IgG1, CD4 immuno-adhesins or soluble rCD4 were incubated with U937 cells (2×10^6 cells per tube) for 60 min at 4°C . Specific binding was calculated by subtracting residual nonspecific binding ($<25\%$ of specific binding) which could not be competed out by a 1,000-fold excess of unlabelled human IgG1. **c**, C1q binding analysis was done essentially as described²⁹, except that gp120 coupled to CNBr-activated Sepharose 6B (Pharmacia) was used as the solid support to aggregate CD4 immuno-adhesins or the anti-gp120 mouse mAb. Proteins were adsorbed to gp120 coupled-beads, incubated with varying concentrations of ^{125}I -labelled C1q, and bound and free C1q were then separated by centrifugation through 20% sucrose. Specific binding was determined from the difference in binding in the presence or absence of added antibody or immuno-adhesin. All data analysis was carried out using the Inplot and Scatplot programs (R. Vandlen, Genentech). Scatplot was modified from the Ligand program (P. Muncy, NIH).

may facilitate clearance by receptors in the liver. The charge of the molecule may also be important, as the CD4 portion of 4y1 contributes a net excess of eleven positively charged amino acids on 4y1, but only three on 2y1. This may increase uptake of rCD4 and 4y1 onto anionic surfaces, accelerating their clearance from the circulation.

Fc receptor and complement binding

Two major mechanisms for the elimination of pathogens are mediated by the Fc portion of specific antibodies. Fc activates the classical pathway of complement, ultimately resulting in lysis of the pathogen, whereas binding to cell Fc receptors can lead to ingestion of the pathogen by phagocytes or lysis by killer cells. The binding sites for Fc cell receptors and for the initiating factor of the classical complement pathway, C1q, are found in the constant region of heavy chain²⁶ (the CH2 domain for C1q²⁷ and the region linking the hinge to CH2 for Fc cell receptors²⁸). We aimed to incorporate both of these functions into the immuno-adhesins. We chose the IgG1 subtype to supply the Fc domain because IgG1 is the best compromise between Fc binding, C1q binding, and long half-life. We show below that the immuno-adhesins bind FcR well, but do not bind C1q.

Three types of Fc cell receptors are known to be expressed on a variety of leukocytes. Of these FcRI, principally expressed

on mononuclear phagocytes, is the only one which binds monomeric human IgG1 with high affinity²⁶. We used competition binding analysis with FcRI receptors on the U937 monocyte/macrophage cell line to characterize the Fc receptor binding of 2y1 and 4y1. Direct saturation binding analysis with human IgG1 gave a K_d of $\sim 3 \times 10^{-9}$ M. In competition binding analyses, the two CD4 immuno-adhesins, but not rCD4, bound to Fc receptors on U937 cells to the same extent and with an affinity indistinguishable from human IgG1 (Fig. 3b, Table 1).

We examined the ability of the immuno-adhesins to bind to the first component of the classical pathway of complement, C1q, by saturation binding analysis. Because binding of C1q increases with the aggregation state of the antibody, with an affinity of $\sim 10^{-4}$ for monomers and $\sim 10^{-8}$ for tetramers of IgG²⁶, we first aggregated the immuno-adhesin using gp120 linked to Sepharose. As a positive control, we measured C1q binding to an anti-gp120 mouse IgG2a monoclonal antibody, (which like human IgG1 binds C1q with high affinity²⁹) aggregated by the same gp120-Sepharose. The affinity of the mouse antibody for C1q determined by Scatchard analysis was 1.8×10^{-8} M (Fig. 3c), comparable to that observed for other mouse IgG2a and for human IgG1 antibodies. In contrast, neither immuno-adhesin bound C1q to any detectable extent (Fig. 3c),

Fig. 4 Pharmacokinetics of CD4 immunoadhesins and soluble rCD4. Shown are the mean plasma concentrations (ng ml⁻¹) for 2γ1 (triangles), 4γ1 (squares), and rCD4 (circles) following a single intravenous administration in rabbits. *a*, Time course of plasma clearance over the first 120 minutes; *b*, time course over 8 days after injection of the CD4 analogues.

Methods. Ten female New Zealand white rabbits (Rabbitek, Modesto, California) were injected intravenously (via an ear vein catheter) with a single bolus dose (40 μg kg⁻¹ in a volume of 1 ml) of either rCD4 (*n* = 2), 4γ1 (*n* = 4), or 2γ1 (*n* = 3). Blood samples were obtained from an arterial catheter in the opposite ear; after 24 hours, blood samples were obtained by venipuncture. Plasma concentrations of each protein were determined by an enzyme-linked immunosorbent assay. This capture assay used two antibodies, including an anti-CD4 monoclonal directed against the gp120-binding site (and capable of blocking gp120 binding), and thus provided a sensitive assay for CD4-containing molecules that are still capable of binding gp120. Exponential equations were fitted to the data of individual rabbits using a nonlinear least squares regression program NONLIN84® (Statistical Consultants, Lexington, Kentucky). The concentration (*C*, ng ml⁻¹) versus time (*t*) data for rCD4 were best described by a biexponential equation $C = 541 e^{-1.34t} + 620 e^{-0.0472t}$ where time is in minutes; the average terminal half-life was 14.7 min, and the average clearance was 3 ml min⁻¹ kg⁻¹. The 4γ1 data were best described by a triexponential equation, $C = 546 e^{-2.11t} + 193 e^{-20.5t} + 46.8 e^{-2.54t}$, where time is in hours. The average terminal half-life was 6.7 hours, and the average clearance was 0.91 ml min⁻¹ kg⁻¹. The 2γ1 data were best described by a triexponential equation, $C = 153 e^{-53.2t} + 342 e^{-2.19t} + 183 e^{-0.351t}$, where time is in hours. The average terminal half-life was 48 hours, and the average clearance was 0.039 ml min⁻¹ kg⁻¹.

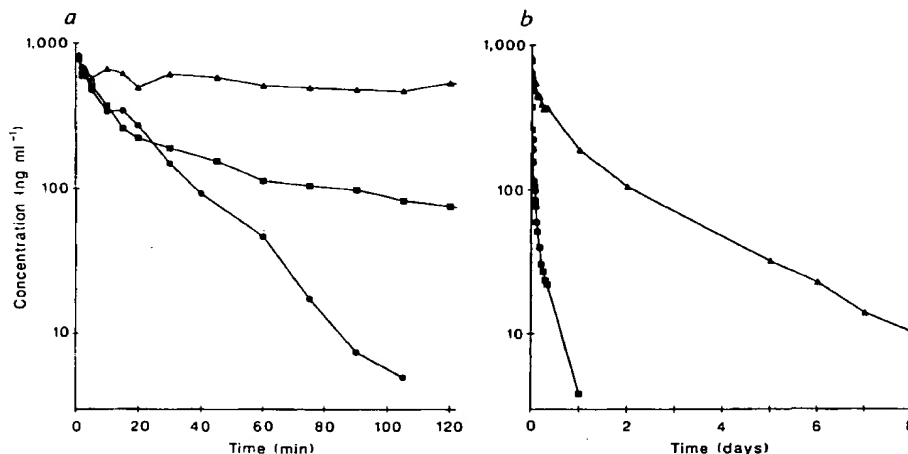
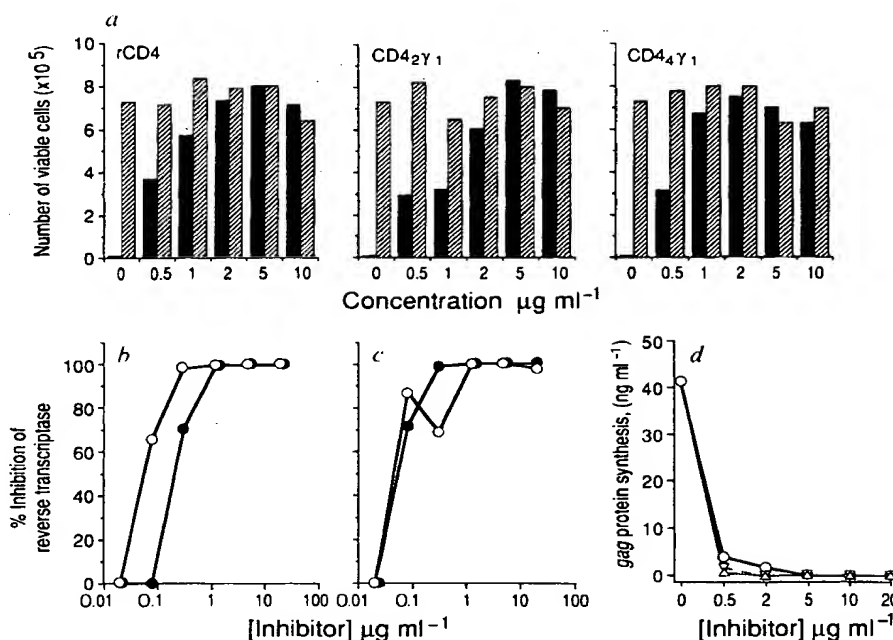


Fig. 5 Inhibition of HIV-1 infectivity by CD4 immunoadhesins and soluble rCD4. *a*, Inhibition of the cytopathic effects on ATH8 cells by HIV-1 was examined as described³² with the HTLV-IIIB isolate³¹. The number of viable cells at day 10 after infection is shown for varying concentrations of each molecule in the presence (solid bars) or absence (shaded bars) of added virus. The absence of an effect of each CD4 analogue on cell number in the absence of virus indicates that none of these molecules inhibited cell growth. *b*, Inhibition of infection of H9 cells by HIV-1 was carried out as described⁷ with the HTLV-IIIB isolate. Reverse transcriptase activity was determined 7 days after infection and is given as the percentage of the level seen in the absence of inhibitor. Solid and open circles represent 2γ1 and 4γ1, respectively. *c*, Inhibition of infection of U937 cells by HIV-1 (HTLV-IIIB isolate) was carried out as described above for H9 cells. *d*, Inhibition of infection of fresh human monocytes by the monocytotropic HIV-1 isolate Ba-L (ref. 35). HIV-1 replication was determined by measuring the level of p24 gag antigen synthesis 10 days after infection using a commercial assay kit (Dupont). Circles, inverted triangles and triangles represent inhibition of p24 synthesis by soluble rCD4, 2γ1 and 4γ1, respectively.



although both did bind the gp120-Sepharose matrix in amounts comparable to the control antibody.

Thus, our immunoadhesins bind well to Fc receptors. It is perhaps surprising that they do not bind C1q. As far as is known, all the critical contact residues for C1q binding reside in the CH2 domain of the heavy chain²⁶, and are conserved among all the human IgG isotypes. However, these have varying abilities to mediate complement fixation. Thus steric hindrance or other aspects of protein conformation (for example, the segmental flexibility of antibodies³⁰) may be important.

Infectivity studies

Two systems were used to study the *in vitro* ability of CD4 immunoadhesins to block infection of CD4-bearing T cells by

the HIV-1 T-lymphotrophic isolate HTLV-IIIB (ref. 31). Infection with HIV-1 exerts a profound cytopathic effect on the human T-cell clone ATH8, with more than 98% of the cells being killed by day 10 after infection³² (Fig. 5a). Both CD4 immunoadhesins blocked cell killing with the same potency as soluble rCD4, without inhibiting cell proliferation; each CD4 analogue completely abolished cell killing at a concentration of ~0.05 μM (Fig. 5a). Complete protection was also observed at comparable concentrations with a different HIV-1 isolate, HTLV-III RF, which is not neutralized by sera from animals immunized with rgp120 from the IIIB isolate⁵. We also examined the production of HIV-1 reverse transcriptase activity after infection of the H9 human T-cell line. Again, both immunoadhesins completely blocked virus production by day 7 (Fig. 5b), at

concentrations comparable to rCD4 (data not shown); moreover the potency of each CD4 analogue was markedly higher (~fivefold) than that observed in the ATH8 assay.

Monocyte infection

Because it has been suggested that antibodies present in sera from HIV-1 infected individuals may enhance the infectivity of HIV-1 in Fc receptor (FcR)-bearing cells such as primary blood monocytes³³, and monocyte cell lines³⁴, we examined the effect of rCD4 and CD4 immunoadhesins on HIV-1 infection of FcR-expressing cells of monocyte/macrophage origin. Both CD4 immunoadhesins completely blocked HIV-1 IIIB virus production in U937 cells at similar concentrations to those found to be effective on H9 cells (Fig. 5c), with a potency comparable to that of soluble rCD4 (data not shown). In another system, the replication of a monocytopathic HIV-1 isolate, Ba-L³⁵, in fresh monocytes was monitored by the production of p24 antigen. Soluble rCD4 completely blocked infection, indicating that infection of monocytes by the Ba-L isolate does involve CD4. Both CD4 immunoadhesins also completely blocked p24 production, at concentrations equal to or lower than rCD4 (Fig. 5d). Thus the CD4 immunoadhesins are at least comparable to soluble rCD4 in their ability to prevent infection of monocyte/macrophages by HIV; no evidence was found for enhancement of infection by immunoadhesins (or by soluble rCD4) in cells which express high affinity Fc receptors.

Implications for treatment of HIV-1 disease

Because the hallmark of HIV-1 disease is the specific destruction of CD4⁺ T cells, and the progression of infected individuals to AIDS closely parallels their decline in CD4⁺ T-cell number³⁶, it is reasonable to believe that the interaction of gp120 with CD4, either by direct HIV-1 infection of CD4⁺ cells or otherwise, underlies the killing of CD4⁺ cells. Therefore, if this interaction can be stopped it may be possible to prevent disease progression. But despite the logic of this hypothesis, the observation that only a very few lymphocytes are actively infected with HIV-1 *in vivo*³⁷ has posed a problem to those attempting to explain the causative role of HIV-1 in the aetiology of AIDS³⁸. Two observations may explain the 'catalytic' ability of HIV-1 to deplete CD4⁺ lymphocytes: first, a single infected cell can fuse many uninfected CD4⁺ cells to itself, creating an inviable mass^{39,40}; and second, gp120 is shed from the surface of HIV-1-infected cells and virions⁴¹, as its link to gp41, its anchor protein partner, is probably non-covalent. This shed gp120 binds to surface CD4 on uninfected cells with high affinity, and can result in their functional alteration^{42,43} or death by one of two pathways shown to operate *in vitro*. Bystander cells coated with gp120 bound to their CD4 surface molecules become targets for anti-gp120 antibodies produced by HIV-1 infected individuals and can be killed via antibody-dependent cell-mediated cytotoxicity⁴⁴. Also, MHC class II-positive CD4⁺ T cells can internalize gp120 bound tightly to CD4 on their surface, process it, and present peptides derived from it on their class II molecules, thus becoming sensitive, even at low gp120 concentrations, to lysis by gp120-specific cytotoxic T cells^{19,45,46}. The important common factor in all these proposed mechanisms of cell destruction is that gp120 must bind specifically to cell-surface CD4. If these mechanisms are important *in vivo*, this would imply that soluble rCD4 could intervene.

But to affect the disease noticeably, one would expect to need to maintain a high concentration of rCD4, which is hampered by its rapid clearance. Our approach to this problem was to fuse the gp120-binding domain of CD4 to a molecule well designed to avoid the clearance mechanisms of the body. Indeed, the Fc domain and CD4 sequences are structurally compatible, as the hybrid molecules have important properties of both parents. Thus, they bind gp120 and block infection of T cells by T-lymphotrophic HIV-1 and of monocytes by monocytopathic HIV-1. They are also comparable to antibodies in

their long plasma half-life and their ability to bind Fc receptors and protein A. This combination of properties allows both a better passive defence, due to the higher plasma concentrations attainable even with infrequent injection, and the possibility of actively attacking HIV-1 and infected cells. A high steady-state level also makes it more likely that effective concentrations will be attained in lymph and lymphatic organs, where HIV may be most active.

The high-affinity binding of the immunoadhesins to Fc receptors implies that mechanisms of pathogen elimination, such as phagocytic engulfment and killing by antibody-dependent cell-mediated cytotoxicity, may be recruited by these immunoadhesins to kill HIV-1 infected cells and virus. As it is possible that antibody-dependent cell-mediated cytotoxicity in an infected individual may be more a mechanism of pathology in HIV-1 infection than a protective response⁴⁴, it is important to note a difference between CD4 immunoadhesins and the patients' own anti-gp120 antibodies: the immunoadhesin, in contrast to antibody, cannot recognize gp120 bound to an uninfected CD4⁺ bystander cell, as gp120 has only a single binding site for CD4. Because placental transfer of antibody, unique to the IgG subclass, also proceeds through an FcR-dependent mechanism, CD4 immunoadhesins may also be transferred *in utero*. This may have implications for the prevention of perinatally transmitted HIV-1 infection.

Although it is not yet clear which of the functions of immunoglobulins will be advantageous when applied to HIV infection, we have taken the approach of trying to add all possible functions to our immunoadhesins. Once the structural requirements for the optimal molecule are established, functions can be tailored at will, as the parent antibody molecule is so well understood.

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LETTERS TO NATURE

A 110-ms pulsar, with negative period derivative, in the globular cluster M15

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We report the discovery of a 110-ms pulsar, PSR2127+11, in the globular cluster M15 (NGC7078)¹. The results of nine months of timing measurements place the new pulsar about 2" from the centre of the cluster, and indicate that it is not a member of a close binary system. The measured negative value of the period derivative, $\dot{P} \approx -2 \times 10^{-17} \text{ s s}^{-1}$, is probably the result of the pulsar being bodily accelerated in our direction by the gravitational field of the collapsed core of M15. This apparently overwhelms a positive contribution to \dot{P} due to magnetic braking. Although PSR2127+11 has an unexpectedly long period, we argue that it belongs to the class of 'recycled' pulsars, which have been spun up by accretion in a binary system. The subsequent loss of the pulsar's companion is probably due to disruption of the system by close encounters with other stars^{2,3}.

The discoveries of millisecond pulsars in globular clusters M28 (ref. 4) and M4 (ref. 5) led us to survey all clusters accessible to the 305-m Arecibo radio telescope ($0^\circ \leq \delta \leq 38^\circ$). A dual-polarization, 40-MHz-bandwidth signal at 1415 MHz was passed through the Arecibo digital correlator, sampled with 128 lags every 506.6 μs , and recorded on tape. The relatively high central radio frequency ensured an almost interference free signal and minimized the effects of interstellar dispersion and scattering, which can be significant for distant, low-galactic-latitude clusters. M15 was observed on 28 December 1987 for 90 minutes, which corresponds to ~ 11 million samples.

The data were analysed at both the Cornell National Supercomputer Facility (IBM 3090-600E) and the Los Alamos National Laboratory (Cray X-MP). Both analyses involved preliminary dedispersion of the multichannel data at 128 or 64 trial dispersion measures, followed first by one-dimensional Fourier transformation of the dedispersed time series and then by a search for harmonically related spikes in the resultant power spectra. The Cray X-MP analysis used the full, 11-million-sample data arrays to obtain maximum sensitivity with regard to isolated pulsars. The data analysed with the IBM supercom-

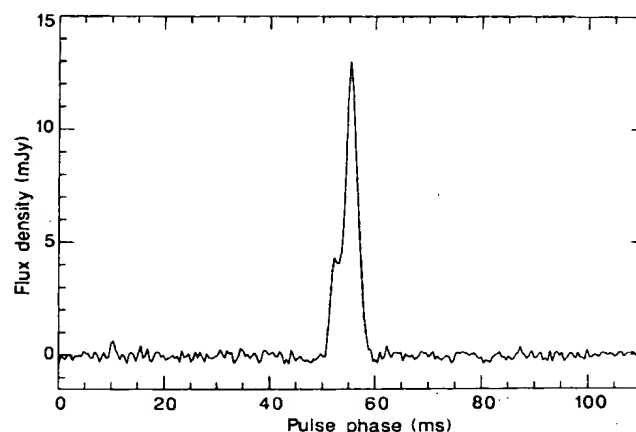


Fig. 1 The average pulse profile of PSR2127+11 at 1415 MHz. The effective resolution is $\sim 800 \mu\text{s}$ and the integration time is 7 hours.

puter were divided into five 2-million-sample blocks, which were treated separately to maintain high sensitivity to binary pulsars with short orbital periods. The nominal 6σ sensitivities of these two analysis schemes were 0.05 mJy and 0.1 mJy respectively, for the periods down to ~ 2.5 ms.

The data analysis at Cornell revealed the presence of a 110-ms, high- Q periodicity in the received signal with dispersion measure $DM \approx 60 \text{ pc cm}^{-3}$. This detection was subsequently confirmed at Los Alamos. Further observations made at Arecibo on 20 and 21 February 1988 confirmed the discovery of a 110-ms pulsar. The average pulse profile of PSR2127+11 observed at 1415 MHz is shown in Fig. 1. The pulsar parameters, derived from our twice-weekly timing observations over nine months, are summarized in Table 1. Errors quoted are the standard 3σ errors of a model fit to the observed pulse arrival times.

Although the precise timing and Very Large Array (VLA) positions of PSR2127+11 will become known soon, the present positional accuracy is sufficient to conclude that the pulsar is located well within the $6''$ core radius of the cluster, $2.0''$ west and $0.6''$ north of the centre⁶. The dispersion measure of PSR2127+11, $DM = 67.25 \text{ pc cm}^{-3}$, agrees well with that expected from a simple model of the galactic electron density distribution⁷, given the distance, $D = 9.7 \text{ kpc}$, and galactic coordinates,

Table 1 Measured parameters of the pulsar PSR2127+11

Pulsar period	$0.11066470954 \pm 0.00000000001 \text{ s}$
Period derivative	$(-20 \pm 1) \times 10^{-18} \text{ s s}^{-1}$
Epoch	JD 2447213.15
Dispersion measure	$67.25 \pm 0.05 \text{ pc cm}^{-3}$
Flux density (430 MHz)	$1.7 \pm 0.4 \text{ mJy}$
Flux density (1400 MHz)	$0.2 \pm 0.05 \text{ mJy}$
Right Ascension (B1950.0)	$21^{\text{h}}27^{\text{m}}33.22^{\text{s}} \pm 0.01$
Declination (B1950.0)	$11^{\circ}56'49.4'' \pm 0.3$
Distance	9.7 kpc

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A Domain in TNF Receptors That Mediates Ligand-Independent Receptor Assembly and Signaling

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A conserved domain in the extracellular region of the 60- and 80-kilodalton tumor necrosis factor receptors (TNFRs) was identified that mediates specific ligand-independent assembly of receptor trimers. This pre-ligand-binding assembly domain (PLAD) is physically distinct from the domain that forms the major contacts with ligand, but is necessary and sufficient for the assembly of TNFR complexes that bind TNF- α and mediate signaling. Other members of the TNFR superfamily, including TRAIL receptor 1 and CD40, show similar homotypic association. Thus, TNFRs and related receptors appear to function as preformed complexes rather than as individual receptor subunits that oligomerize after ligand binding.

Tumor necrosis factor (TNF- α) is an important effector cytokine for immune responses and inflammation (1). TNF- α exerts its biological effects through two TNF receptors (TNFRs): a 60-kD receptor (p60) and an 80-kD receptor (p80). The TNFRs are the prototypes of a large family of cell surface receptors that are critical for lymphocyte development and function (2). Homotrimeric TNF- α is thought to recruit three receptor chains into a complex that juxtaposes the cytoplasmic domains (CDs). Subsequently, p60 recruits apoptosis-inducing and other proteins through a "death domain" in its cytoplasmic tail, whereas p80 induces inflammatory responses through a cytoplasmic TNFR-associated factor (TRAF)-binding domain (3). Signaling may also require loss of binding of cytosolic negative regulators such as the Silencer of Death Domain (SODD) protein (4). The extracellular domain (ECD) of both TNFRs contains three well-ordered cysteine-rich domains (CRD1, -2, and -3) that characterize the TNFR superfamily and a less conserved, membrane-proximal, fourth CRD (5). The ligand-binding pocket for TNF- α is mainly formed by CRD2 and CRD3 of the TNFRs (5). How CRD1 contributes to receptor function is unknown.

Because the first step in signaling by members of the TNFR superfamily is thought to be ligand-induced trimerization of the re-

ceptor (6), we attempted to identify trimer complexes using the thiol-cleavable, membrane-impermeant, chemical crosslinker 3,3'-dithiobis[sulfosuccinimidylpropionate] (DTSSP) (7). Indeed, complexes were found for p80 that exhibited molecular sizes approximately three times the unit size, consistent with glycosylated and nonglycosylated trimers (Fig. 1A). The p80 complexes were efficiently captured in the presence or absence of TNF- α (65 to 70% by densitometry). Despite the fact that most p60 resides in the Golgi apparatus and was inaccessible to the cross-linker (8), as much as 15 to 20% of the p60 chains were cross-linked as apparent trimers and discrete higher order complexes, whether or not TNF- α was added (Fig. 1A). Control experiments detected no endogenous TNF- α and no other proteins such as p80 cross-linked to the p60 complex (9). The complexes were reduced to monomers by cleaving the cross-linker with β -mercaptoethanol (Fig. 1A).

Because p60 and p80 chains apparently self-associate before ligand binding, we sought a domain that would mediate ligand-independent self-assembly. It is well established that the cytoplasmic death domain of p60 can self-associate and trigger apoptosis when overexpressed (10). However, because the preassembled complexes we observed were apparently nonsignaling, we hypothesized that the assembly domain resides outside of the cytoplasmic region. Indeed, the NH₂-terminal regions of the ECDs of p60 and p80 could specifically self-associate in a yeast two-hybrid interaction assay (11). In mammalian cells, a chimeric p60 receptor with the CD replaced by the green fluorescent protein (GFP) interacted strongly with a CD-

deleted p60 (p60 Δ CD-HA) but not with the TNFR-like herpesvirus receptor (HveA Δ CD-HA) (Fig. 1B) (12). GFP alone failed to associate with p60 Δ CD-HA (Fig. 1B). Homotypic interaction was also observed between full-length p80 and p80 Δ CD-HA (Fig. 1C). However, removal of amino acids 10 through 54 of p80, overlapping CRD1, completely abrogated association with intact p80 (Fig. 1C). Self-association was eliminated by a similar deletion (amino acids 1 through 54) in p60 (13).

The importance of the NH₂-terminus of p80 (amino acids 10 through 54) was further illustrated by experiments in which it was appended to the p60 receptor. This chimeric receptor interacted with full-length p80 (Fig. 1D). Thus, this domain was sufficient to mediate specific association of a heterologous receptor. This association is ligand-independent because the chimera p80₁₀₋₅₄p60₅₅₋₂₁₁(R1)-HA has two amino acids encoded by an Eco RI restriction site inserted at the junction of the p80 and p60 sequences that abolished TNF- α binding but permitted self-association (Fig. 1E). Thus, a distinct functional domain of the TNFR-ECD mediates self-assembly in the absence of ligand. Henceforth, we refer to this as the pre-ligand-binding assembly domain (PLAD).

The deletion of the PLAD from either p60 or p80 completely abrogated ligand binding (Table 1 and Fig. 1E) but was unlikely to disrupt the overall ECD structure (14). However, the addition of the PLAD from p80 enabled the PLAD-deleted p60 (p80₁₀₋₅₄p60₅₅₋₂₁₁-HA) to bind TNF- α (Fig. 1E). Thus, efficient TNF- α binding by TNFRs depends on receptor self-assembly. Furthermore, two substitutions (15) in the PLAD that are not expected to disturb direct ligand contact, Lys¹⁹ Tyr²⁰ → Ala¹⁹Ala²⁰ (KY19/20AA) and Lys³² → Ala³² (K32A) (5), abrogated self-association (Fig. 2A) and eliminated TNF- α binding (Table 1). Substitution of another residue within the PLAD, Q24A (16), did not affect self-association or TNF- α binding (Fig. 2A and Table 1). In contrast, two substitutions outside of the PLAD in the CRD2 ligand binding pocket, E57A and N66F, disrupted TNF- α binding but had little effect on receptor self-association (Table 1 and Fig. 2A). Association of a mutant receptor lacking the CD with the wild-type ECD correlated with its ability to dominantly interfere with p60-induced apoptosis, indicating that the mutant receptors enter into endogenous functional p60 receptor complexes via the PLAD (Table 1). Thus, the PLAD is physically distinct from the ligand contact domain but is nonetheless essential for efficient TNF- α binding and receptor function.

To confirm receptor self-interaction in living cells, we used a flow cytometric approach to analyze fluorescence resonance energy

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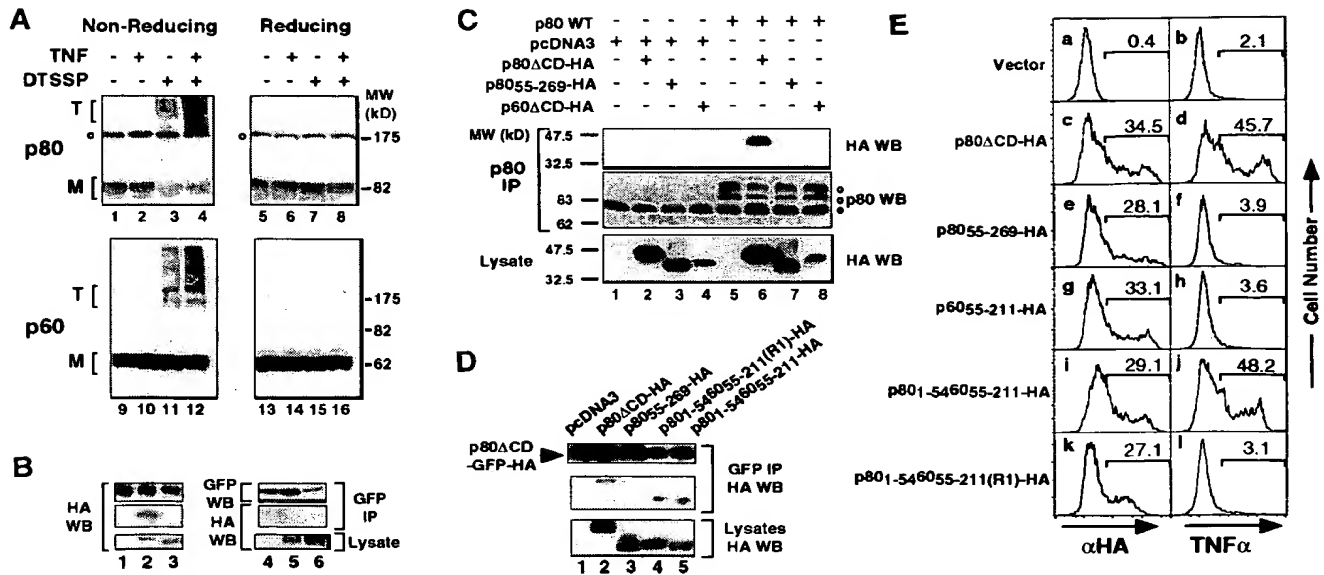


Fig. 1. Definition of the PLAD. **(A)** Trimeric TNFR complexes in the absence of ligand. H9 cells were treated as indicated and analyzed for p60 or p80 complexes on Western blot (WB). The position of monomers (M), trimers (T), and a nonspecific protein species (open circle) are shown. **(B)** Specific self-association of p60. 293T cells were transfected with p60ΔCD-GFP-HA (lanes 1 through 3) or pEGFP-N1 (lanes 4 through 6) and with pcDNA3 (lanes 1 and 4), p60ΔCD-HA (lanes 2 and 5), or HveAΔCD-HA (lanes 3 and 6). Immunoprecipitation (IP) (top two panels)

and WB (bottom panel) are shown (12). **(C)** Specific self-association of p80. IP and WB were done as shown. The glycosylated and unglycosylated forms of p80 (open circles) and IgH (solid circle) are indicated. **(D)** The PLAD is necessary and sufficient for self-association. Cotransfection of p80ΔCD-GFP-HA (lanes 1 through 5) with the indicated plasmids is shown. IP and WB are shown. **(E)** The PLAD is required for TNF-α binding (26). The numbers shown are percentages of positive population compared to the vector-transfected control.

Table 1. Summary of the phenotypes of the p60ΔCD mutants (16).

	MAB225*	Clone 4.12†	TNF-α binding‡	Self-association§	Dominant interference
p60ΔCD	1	1	1	+	+
p60 ⁵⁵⁻²¹¹	0.87	0.02	0.01	NT	—
K19E	1.05	1.07	1.1	NT	+
KY19/20AA	0.59	0.14	0.03	—	—
Q24A	1.06	0.97	1.13	+	+
K32A	0.36	0.01	0.01	—	—
DT49/50AA	1.16	0.96	1.13	NT	+
E57A	1.68	0.04	0.02	+	+
T61A	1.42	1.28	1.35	NT	+
N66F	0.67	0.04	0.01	+	+
R77A	1.06	0.99	1.24	NT	+
W108T	1.32	1.11	1.25	NT	+
L112E	1.31	1.27	0.85	NT	+

*Staining of p60-specific monoclonal antibody clone MAB225 (R&D Systems). The values were normalized against the staining of the HA epitope tag by dividing the percentage of MAB225-positive cells by the percentage of HA-positive cells. †Staining with p60-specific monoclonal antibody clone 4.12 (Zymed) was normalized against HA staining (26). ‡TNF-α binding was determined with a biotinylated form of TNF-α and normalized against HA staining (26). §Self-association was determined by immunoprecipitation assays in 293T transient transfections as described (12). NT, not tested. ||Dominant interference was determined as described (27). Dominant inhibition by the p60ΔCD-HA mutants was at least 50% of p60ΔCD wild type (+). p60⁵⁵⁻²¹¹, KY19/20AA, and K32A did not confer any protection (<5%) against TNF-induced death (—) in all experiments. The antibodies and TNF-α binding to p60ΔCD are arbitrarily set at 1. Results are representative of three independent experiments.

transfer (FRET) (17) between receptor subunits fused at the COOH-terminus to either cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) (18) as described in an accompanying paper [also see the protocol at *Science's* STKE (www.stke.org/cgi/content/full/OC_sigtrans;2000/38/pl1)] (19). We found that there was energy transfer between p60ΔCD-CFP and p60ΔCD-YFP that increased substantially after the addition of TNF-α (Fig. 2B). This FRET was abolished by

deletion of the PLAD or by the K32A mutation that prevented PLAD association (Fig. 2B). The p80ΔCD-CFP:p80ΔCD-YFP pair also yielded a strong FRET signal that increased with TNF-α addition (Fig. 2B). Controls using p60ΔCD-YFP as an acceptor for p80ΔCD-CFP or CFP-p80ΔCD (CFP fused to the NH₂-terminus of p80 ECD) as donor showed no FRET (Fig. 2B). Thus, the p60 and p80 chains are in close proximity to themselves in living cells, and ligand induces a change in the complexes

that leads to tighter association of the CFP and YFP moieties in the cytoplasm. Furthermore, other members of the TNFR superfamily, including the ECDs of TRAIL receptor 1 (DR4), CD40 (Fig. 2, C and D), and Fas (19, 20), all self-associate but do not interact with ECDs from heterologous receptors. Thus, self-assembly through the PLAD is a conserved feature of the TNFR superfamily.

The presence of PLAD-mediated pre-assembled TNFR complexes sheds new light on signaling by this large family of receptors, many of which are critical for lymphocyte function and homeostasis (2). Previously, ligand was thought to bring monomer receptor chains into apposition in threefold complexes that recruit cytoplasmic signal transduction proteins (1, 3, 5, 6). It is now clear that p60 and p80 preassociate as oligomers on the cell surface and are only found as monomers if the PLAD is deleted. Cross-linking the endogenous p60 and p80 receptors suggests that trimers are a favored conformation. However, the p60 ECD crystallizes in the absence of ligand as parallel dimer (21), which suggests that further work will be needed to define the stoichiometry of cell-surface oligomers. The presorting of chains into homotypic complexes on the cell surface could promote the rapidity and specificity of response for the different receptors in the TNFR superfamily (3). Also, "receptor interference" in which, for example, a p80 chain (lacking a death domain) is recruited by TNF-α into a complex with p60 and causes dominant inhibition

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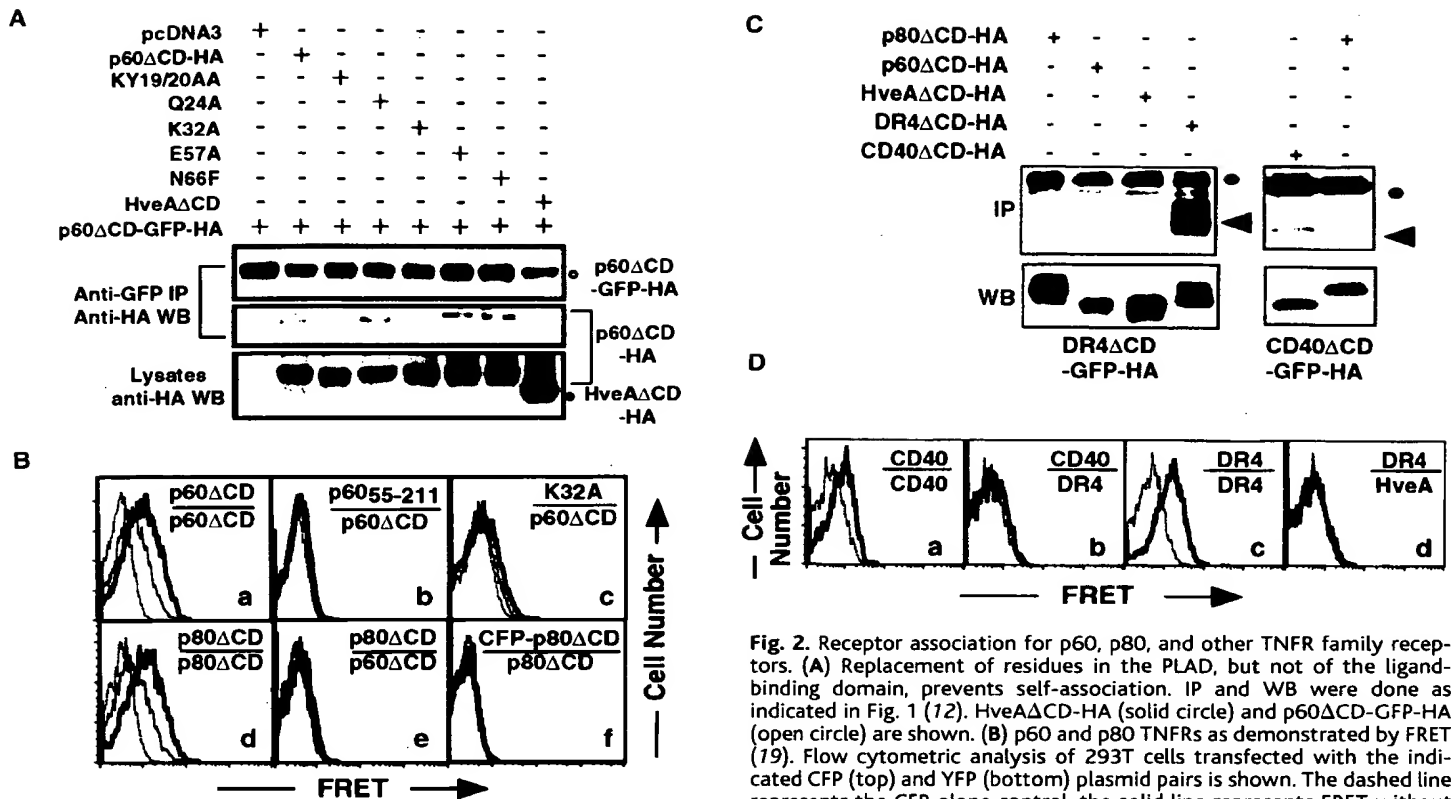


Fig. 2. Receptor association for p60, p80, and other TNFR family receptors. (A) Replacement of residues in the PLAD, but not of the ligand-binding domain, prevents self-association. IP and WB were done as indicated in Fig. 1 (72). HveAΔCD-HA (solid circle) and p60ΔCD-GFP-HA (open circle) are shown. (B) p60 and p80 TNFRs as demonstrated by FRET (19). Flow cytometric analysis of 293T cells transfected with the indicated CFP (top) and YFP (bottom) plasmid pairs is shown. The dashed line represents the CFP-alone control, the solid line represents FRET without TNF-α, and the thick line represents FRET with TNF-α added. (C) Self-association of CD40 and DR4. IP and WB were performed with antibodies to GFP and HA, respectively. The solid circles denote the GFP fusion proteins, and the arrowheads indicate the ΔCD protein in the immune complexes. (D) Specific receptor association of DR4 and CD40 as demonstrated by FRET. Transfections with the indicated CFP (top) and YFP (bottom) plasmid pairs were performed as in (B). The dashed lines represent background FRET with CFP alone, and the thick lines represent FRET in the presence of both CFP and YFP fusion proteins.

of apoptosis would be avoided (22, 23). Pre-assembly has been described for other receptor families, notably interleukin-1 (IL-1) and IL-2 receptor, which are composed of heteromers of different polypeptides (24). The erythropoietin receptor dimers apparently undergo a scissors-type movement to accommodate ligand (25). In that case, self-association of the receptor chains occurs via the same amino acid contacts that are critical for ligand binding (25). By contrast, the TNFR superfamily uses a dedicated self-association domain distinct from the CRD2/3 ligand contact region. Identification of the PLAD could allow development of therapeutics that selectively inhibit the PLAD of individual TNFR-like receptors and thereby prevent signaling.

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7. H9 lymphoma cells were washed and resuspended in phosphate-buffered saline. The cells were then incubated with human recombinant TNF-α (100 ng/ml) (R&D Systems) for 1 hour at 4°C with rotation. Cells were then treated with a 2 mM solution of the cross-linker DTSSP (Pierce) for 30 min, and the reaction was quenched with 20 mM Tris-Cl (pH 7.5) for 15 min on ice. The cells were lysed in 150 mM NaCl, 20 mM Tris-Cl (pH 7.5), 1 mM EDTA, 30 mM NaF, 2 mM β-glycerophosphate, and 1 mM sodium orthovanadate with protease inhibitors added (Boehringer-Mannheim). Equal amounts of the lysates were subjected to electrophoresis under nonreducing (without β-mercaptoethanol) or reducing (with 280 mM β-mercaptoethanol) conditions and were analyzed for p60 and p80 complexes with specific antibodies (Santa Cruz). Densitometry was performed with a Kodak Image Station 440. Results shown are representative of three independent experiments.
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9. Western blot analysis confirmed the absence of TNF-α in the lysates. Immunoprecipitation of the cross-linked complexes with antibody to p60 revealed no detectable level of p80 in the p60 complex in Western blots.
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12. The various truncations and mutations of p60, p80, HveA, DR4, and CD40 were generated by polymerase chain reaction (PCR) and sequenced. Briefly, the leader sequence and the first 10 amino acid residues from p80 were amplified so that the hemagglutinin (HA) epitope tag was included at the 3' end to create a HA tag at the NH₂-terminus of the receptors. The PCR product was digested with Bam HI and Eco RI and cloned into pcDNA3. The PCR fragments containing the receptor fragments were then introduced into this plasmid using the Eco RI and Xho I sites. For the GFP/CFP/YFP chimeras, the fragments were amplified by PCR and introduced in-frame into the Xho I and Xba I sites of p60ΔCD-HA. 293T cells were transfected with Fugene 6 (Boehringer-Mannheim) as per the manufacturer's protocol. Cells were lysed in 150 mM NaCl, 20 mM Tris-Cl (pH 7.5), 1 mM EDTA, 30 mM NaF, 2 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM iodoacetamide, 2 mM dithiothreitol, 1% Triton X-100, and protease inhibitors (Boehringer-Mannheim). After preclearing with protein G agarose beads (Boehringer-Mannheim) and normal mouse immunoglobulin G (IgG), proteins were immunoprecipitated from the lysates with 2 μg of anti-GFP and protein G agarose beads. Immune complexes were washed twice with lysis buffer containing 0.5 M NaCl and then three times with regular lysis buffer. Immune complexes were resolved on Tris/Glycine gels (Novex, San Diego, CA). Results shown are representative of five independent experiments. Transfection in Jurkat cells showed similar results.
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15. Mutagenesis was performed with the Quikchange method (Stratagene) as per the manufacturer's instructions. The mutations were confirmed by DNA sequencing.
16. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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26. Receptor expression was monitored by staining with monoclonal antibodies to HA and p80 (clone MAB226, R&D Systems). TNF- α binding was determined with biotinylated TNF- α (NEN Life Sciences) and a secondary fluorescein-conjugated streptavidin label. Samples were analyzed on a FACScan flow cytometer. Results shown are representative of at least five experiments.
27. Fifteen micrograms of the corresponding plasmids

were transfected into p80 Jurkat cells (22) by electroporation using a BTX Electro Cell Manipulator 600. After 9 to 24 hours, cells were stimulated with the indicated amount of TNF- α for 12 to 16 hours. Cells were then stained for HA expression and propidium iodide uptake. The number of HA-positive cells was scored under constant time, and percent inhibition of apoptosis was calculated by normalizing to the percentage of cell death induced in the HveA Δ CD4A-transfected samples. Results are representative of three experiments.

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Fas Preassociation Required for Apoptosis Signaling and Dominant Inhibition by Pathogenic Mutations

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Heterozygous mutations encoding abnormal forms of the death receptor Fas dominantly interfere with Fas-induced lymphocyte apoptosis in human autoimmune lymphoproliferative syndrome. This effect, rather than depending on ligand-induced receptor oligomerization, was found to stem from ligand-independent interaction of wild-type and mutant Fas receptors through a specific region in the extracellular domain. Preassociated Fas complexes were found in living cells by means of fluorescence resonance energy transfer between variants of green fluorescent protein. These results show that formation of preassociated receptor complexes is necessary for Fas signaling and dominant interference in human disease.

Fas (CD95 or APO-1) is a cell surface receptor that transduces apoptotic signals critical for immune homeostasis and tolerance (1–3). The Fas protein is a 317-amino acid type 1 transmembrane glycoprotein with three extracellular cysteine-rich domains (CRDs) that are characteristic of the tumor necrosis factor receptor (TNFR) superfamily. Both Fas and Fas ligand (FasL) are predicted to form trimers, with CRD2 and CRD3 forming the major

contact surfaces for FasL (4, 5). The Fas cytoplasmic portion contains a death domain that rapidly recruits the adaptor molecule FADD (Fas-associated death domain protein) and the caspase-8 proenzyme after binding of FasL or agonistic antibodies, leading to caspase activation and apoptosis (6–10).

Patients with autoimmune lymphoproliferative syndrome (ALPS) type 1A have heterozygous germ line mutations in the *APT-1* Fas gene. Their lymphocytes are resistant to Fas-induced apoptosis, and transfection of the mutant allele causes dominant interference with apoptosis induced through Fas (11–16). This was thought to result from ligand-mediated crosslinking of wild-type and defective Fas chains into mixed trimer complexes. However, a mutation that causes an

extracellular domain deletion of most of CRD2 (ALPS Pt 2, deletion of amino acids 52 to 96) as a result of altered RNA splicing shows no binding to agonistic antibodies or FasL, but still dominantly interferes with Fas-induced apoptosis almost as efficiently as does a death domain mutant [ALPS Pt 6, Ala²⁴¹ \rightarrow Asp (A241D)] (Fig. 1A) (13, 17). Control experiments showed equal cell surface expression of the wild-type and mutant Fas molecules (18). Thus, dominant interference cannot be explained by the conventional model of signaling by FasL-induced oligomerization of receptor monomers because, in this scheme, the Pt 2 mutant Fas molecule would not become part of a mixed receptor complex. We therefore tested for ligand-independent interactions between Pt 2 Fas and wild-type Fas. Both full-length and Pt 2 Fas coprecipitated with a Fas 1–210:GFP chimera in which green fluorescent protein (GFP) replaces the death domain (Fig. 1C). This interaction was specific, because the TNFR family receptors TNFR2/p80 and HveA did not interact with Fas (1).

We have found that TNFR superfamily members share a self-association domain in CRD1, termed the "pre-ligand assembly domain" (PLAD) (Fig. 1B) (19). To test whether Fas contains a functional PLAD, we constructed hemagglutinin (HA)-tagged NH₂-terminal Fas truncations (20). Deleting the first subdomain in CRD1 (amino acids 1 to 42) (21) substantially reduced ligand binding but did not prevent binding of the Fas monoclonal antibody (mAb) APO-1. Deleting the entire CRD1 (amino acids 1 to 66) abrogated binding of both FasL and Fas mAb (Fig. 1A). Both truncations eliminated coprecipitation with a differentially tagged Fas molecule and abrogated apoptosis signaling; this result indicates that the NH₂-terminus of Fas, including CRD1, functions as a PLAD (Fig. 1, C

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Original Contributions

TWO HUMAN TNF RECEPTORS HAVE SIMILAR EXTRACELLULAR, BUT DISTINCT INTRACELLULAR, DOMAIN SEQUENCES

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Tumor necrosis factor (TNF) is a cytokine with a wide range of biological activities in inflammatory and immunologic responses. These activities are mediated by specific cell surface receptors of 55 kDa and 75 kDa apparent molecular masses. A 75-kDa TNF receptor cDNA was isolated using partial amino acid sequence information and the polymerase chain reaction (PCR). When expressed in COS-1 cells, the cDNA transfers specific TNF-binding properties comparable to those of the native receptor. The predicted extracellular region contains four domains with characteristic cysteine residues highly similar to those of the 55-kDa TNF receptor, the nerve growth factor (NGF) receptor, and the CDw40 and OX40 antigens. The consensus sequence of the TNF receptor extracellular domains also has similarity to the cysteine-rich sequence motif LIM. In marked contrast to the extracellular regions, the intracellular domains of the two TNF receptors are entirely unrelated, suggesting different modes of signaling and function.

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Tumor necrosis factor (TNF) is a highly potent cytokine. Its wide range of biological activities in inflammatory and immunologic responses have triggered many studies of the specific cell surface receptors that mediate TNF function.¹⁻¹⁰ TNF receptors of significantly different molecular masses in the range of 50 to 140 kDa were reported in protein cross-linking studies by various investigators; the possibility that more than one receptor existed therefore had to be considered. We have identified and purified from human cell lines and placenta two distinct human TNF receptors of 55 kDa and 75 kDa that are simultaneously expressed to different extents by various cells.^{8,11,12} Both receptors bind TNF- α and TNF- β with high affinity^{11,13} (also, Schoenfeld and Loetscher, unpublished data). A third TNF-binding protein of 65 kDa was found by SDS-polyacrylamide gel electrophoresis (PAGE) and ligand blotting to copurify

with the 75-kDa receptor fraction from HL60 cells. Both the 75-kDa and 65-kDa proteins in Western blots bind the same monoclonal antibody, utr-1.¹¹ We therefore assume the 65-kDa protein to be a derivative or fragment of the 75-kDa receptor and refer to the two proteins as the 75-kDa receptor.

The cDNA cloning of the 55-kDa receptor has been reported^{14,15}; the open reading frame of the cDNA predicts a receptor protein with extracellular, transmembrane, and intracellular regions. A surprisingly high degree of sequence similarity to the nerve growth factor (NGF) receptor extracellular region was discovered which is most clearly delineated by a repetitive cysteine residue pattern. Recently, the cDNA of the 75-kDa TNF receptor was identified in a eukaryotic expression cloning system.¹⁶ We have independently isolated a 75-kDa TNF receptor cDNA using peptide sequencing and PCR techniques which confirms the sequence reported for the cDNA isolated by expression cloning.¹⁶ When expressed in COS-1 cells, the cDNA transfers specific TNF-binding properties comparable to those of the native receptor. The predicted extracellular region contains four domains with characteristic cysteine residues highly similar to that of the 55-kDa TNF receptor and to that of the NGF receptor,^{17,18} CDw40,¹⁹ and OX40 antigen²⁰ extracellular domains. The intracellular domains of the two TNF receptors, however, are entirely unrelated. We therefore propose that the two TNF

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receptors may address distinct intracellular signal trans-mission pathways.

RESULTS

Isolation of the 75-kDa TNF Receptor cDNA

The 75-kDa and 65-kDa protein bands of the 75-kDa TNF receptor from a preparative SDS-polyacryl- amide gel were blotted onto PVDF membrane and subjected to NH₂-terminal amino acid sequencing by gas phase sequenation as reported elsewhere.¹² Briefly, two parallel sequences were obtained with the 65-kDa band; since one sequence matched the ubiquitin se- quence, the unique sequence could be identified as LPAQVAFTPYAPEPGSTC.¹² Furthermore, the amino acid sequences of a total of seven internal peptides

prepared by tryptic and proteinase K digests of the 75-kDa receptor fraction were determined. The four peptide sequences used in the isolation of the cDNA clone are indicated in Fig. 1; the remaining three peptides, i.e. L¹¹⁴—P¹¹⁷, P¹²³—V¹³⁷ and G²⁸⁸—P³⁰², match the predicted amino acid sequence and thus confirm that the cDNA encodes the receptor. To prepare a probe for the isolation of cDNA clones a short DNA fragment was amplified by polymerase chain reaction (PCR) from human genomic DNA with the use fully degenerate primer oligonucleotides derived from the Q⁴¹²—L⁴²⁸ amino acid sequence (see Fig. 1 and Materi- als and Methods). A DNA fragment of the predicted size was found to be amplified by PCR. Oligonucleotides were synthesised according to the sequence of this DNA fragment and used to identify the cDNA shown in Fig. 1

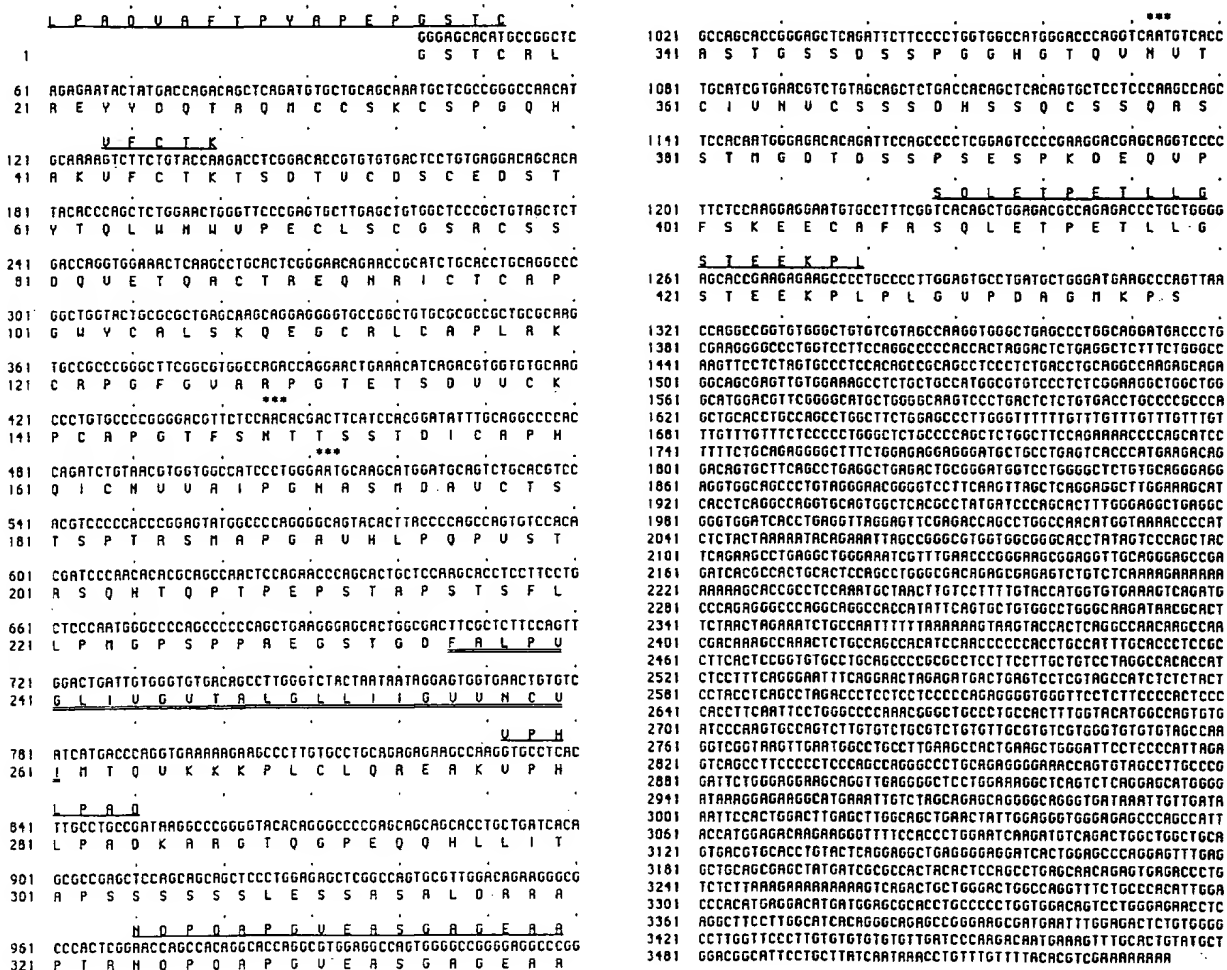
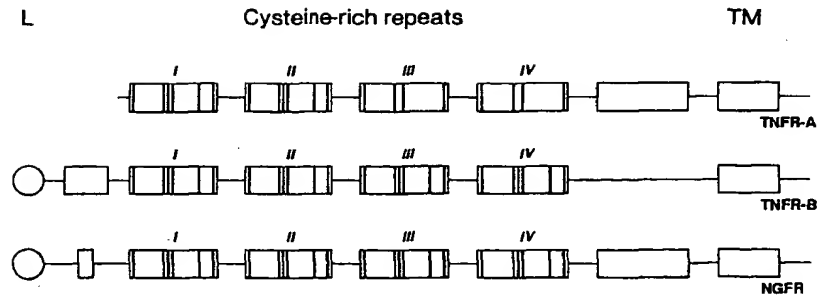


Figure 1. Amino acid sequences of the NH₂ terminus and internal tryptic peptides, and the cDNA nucleotide and predicted amino acid sequences of the 75/65-kDa TNF receptor.

Amino acid sequences determined by protein sequencing are underlined. The amino acid sequence starts at the 65-kDa receptor NH₂ terminus. The predicted transmembrane domain is doubly underlined. Potential N-linked glycosylation sites are marked by asterisks.

Figure 2. Schematic representation of the domain structure of the extracellular regions of the two TNF receptors and of the NGF receptor.

The domains are boxed. Cysteine residues are represented by vertical lines. The domain boundaries correspond to amino acid residues of Fig. 1: residues 17 to 54 (domain I), 55 to 97 (II), 98 to 140 (III), and 141 to 179 (IV). TNFR-A, 75-kDa TNF receptor; TNFR-B, 55-kDa TNF receptor; NGFR, NGF receptor^{17,18}; L and TM, predicted leader and transmembrane regions, respectively.



in cDNA libraries prepared from HL60 and placenta. This cDNA has an open reading frame that predicts a 439-amino acid membrane protein with extracellular (235 residues), transmembrane (26 residues), and intracellular (178 residues) regions. Three basic amino acids are located in the intracellular region sequence adjacent to the putative inner membrane face.

In the predicted amino acid sequence of the extracellular region of the 75-kDa TNF receptor four conserved domains were discovered which are most clearly delineated by a repetitive pattern of cysteine residues schematically represented in Fig. 2. The first two domains contain six cysteine residues in a CX₁₂₋₁₄CX₀₋₂CX₂₋₃CX₇₋₉CX₇C pattern which is highly homologous to that of the four domains of the previously reported 55-kDa TNF receptor extracellular region with the consensus sequence CX₁₀₋₁₅CX₀₋₂CX₂CX₅₋₁₁CX₃₋₈C.^{14,15} In the third and fourth domains of the 75-kDa receptor this cysteine pattern is less well conserved, but the alignment of the total extracellular regions of the two TNF receptors scores significantly above the random score with the Mutation Data Matrix.²¹ This alignment score establishes a significant sequence similarity between the extracellular domains of the two TNF receptors as well as to those of the NGF receptor and the CDw40 and OX40 antigens.¹⁷⁻²⁰ Furthermore, we note that this sequence motif has some similarity to the cysteine-rich, putative metal-binding motif referred to as LIM.²²

In sharp contrast to the high degree of homology between the extracellular domains, the intracellular regions of the two TNF receptors do not exhibit any recognizable sequence similarity. A search of amino acid sequence data banks with the 75-kDa receptor

intracellular domain sequence revealed no significant similarity to other known mammalian sequences. The intracellular regions of both TNF receptors are rich in proline and serine residues (75-kDa receptor: 18% Ser, 9% Pro; 55-kDa receptor: 8% Ser, 12% Pro). Similar proline/serine-rich structures have been found in the intracellular regions of several growth factor receptors.^{23,24}

TNF Binding in COS-1 Cell Transfectants

To confirm that the cDNA presented in Fig. 1 encodes a TNF-binding cell surface protein, the cDNA was recloned in the pLJ268 expression vector²⁵ and transfected into COS-1 cells; transient transfectants were analysed for ¹²⁵I-TNF binding. Specific TNF-binding properties were conferred to the COS-1 cells by the transfected cDNA (Table 1). Expression of the 75-kDa receptor was confirmed in cell lysates of transfectants with the specific monoclonal antibody utr-4¹¹ (Table 1). TNF binding was also studied with COS-1 cell transfectants at various ligand concentrations and the binding data were analysed according to Scatchard (Fig. 3). The transfected cells were found to express a TNF-binding protein characterized by a K_d of about 0.1 nM, which is clearly distinct from the endogenous lower-affinity TNF receptor of COS-1 cells.¹⁴ An analysis of the COS-1 cell transfectants in the fluorescence microscope after staining with the 75-kDa TNF receptor-specific monoclonal antibody utr-1 revealed that only a very small percentage of the cells expressed receptor. The cause of the apparently low transfection

TABLE 1. TNF binding and expression of TNF receptor protein in COS-1 cell transfectants

Transfectant	Specific cell surface bound TNF-α		Relative expression of 75/65-kDa versus 55-kDa receptor in cell lysate†
	cpm/dish*	cpm/10 ⁶ cells	
Specific DNA COS-1 cell transfectant	5,170	890	1.39
Control DNA COS-1 cell transfectant 1§	1,230	210	0.05
Control DNA Cos-1 cell transfectant 2§	1,010	185	0.15

*All values are the average of two independent experiments.

†The quotient of specific 75-kDa and 55-kDa receptor ¹²⁵I-TNFα binding measured in sandwich assays (see Materials and Methods).

§Controls 1 and 2 refer to parallel transfectants in which constructs in which the cDNA was ligated into the expression vector in a false reading frame were used.

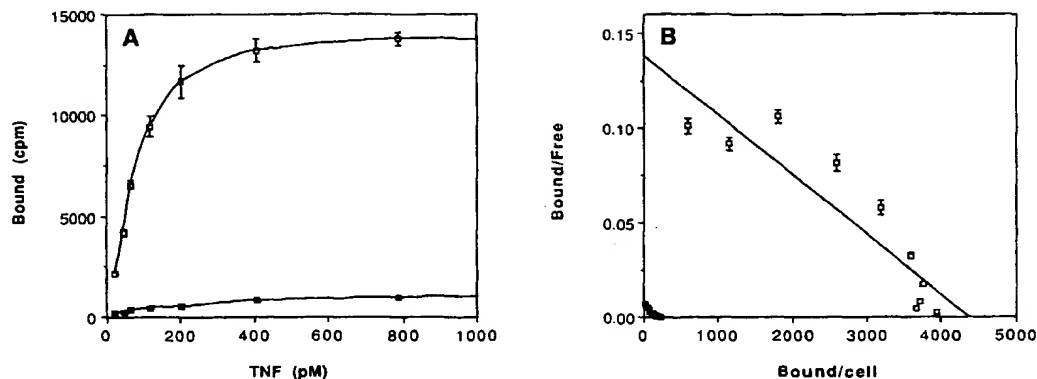


Figure 3. ^{125}I -TNF- α binding to transient COS-1 cell transfectants.

(A) Specific binding at various TNF- α concentrations. Measurements at higher concentrations confirmed saturation of TNF binding (data not included in figure). (B) Plot of the binding data according to Scatchard. The mean and standard deviations of triplicate experiments are given. The assays with transfected and control cells contained 2.2×10^6 and 4.3×10^6 cells/assay, respectively. □, 75-kDa TNF receptor transfectants; ■, non-transfected control cells. The K_d 's of transfected and control cells from Scatchard analysis are about 0.1 and 0.2 nM, respectively.

yield remains unknown, but it explains the low receptor copy number in the pool of transiently transfected cells.

TNF Receptor Expression in Cell Lines

The expression of the TNF receptors was studied in human cell lines by Northern analyses (Fig. 4). Previous flow cytometric analyses of cells stained with receptor-specific monoclonal antibodies had shown that HEp2 cells stain for the 55-kDa receptor only, while HL60 cells stain for both the 55-kDa and 75-kDa receptors.¹¹ In agreement with previous reports, Raji cells were found to be devoid of TNF receptors.^{11,26} These findings were supported by the Northern blot analyses shown in Fig. 4. We note, however, that the lack of 75-kDa TNF receptor expression appears not to be a stable property

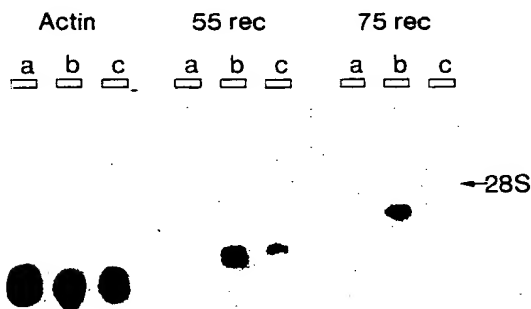


Figure 4. Northern analysis of TNF receptor expression in Raji (a), HL60 (b), and HEp2 (c) cell lines.

By cell surface staining with specific monoclonal antibodies, no TNF receptors are detected on Raji cells, low amounts of 55-kDa receptor (55 rec) are detected on HEp2 cells, and both 55-kDa and 75-kDa receptors (75 rec) are detected at relatively higher levels on HL60 cells.

of Raji cells, since other investigators detect 75-kDa TNF receptor mRNA in these cells.¹⁶ From preliminary studies of 55-kDa and 75-kDa TNF receptor expression HL60 cells appear to be more representative of the average human cell than HEp2 or Raji cells, because many cells were found to express both TNF receptors simultaneously, albeit to very different extents.

Expression of Each TNF Receptor is Independently Regulated

To investigate the regulation of the two TNF receptors, we have studied their expression in phytohemagglutinin-activated peripheral blood lymphocytes (PBL). By cell surface staining with the specific monoclonal antibodies utr-1 (anti-75-kDa receptor) and htr-9 (anti-55-kDa receptor),¹¹ we find that the expression of the 75-kDa receptor is strongly induced from a low resting level, whereas the 55-kDa receptor expression remains at a constant and low level after mitogen activation (Fig. 5). The inducibility of TNF receptors in several cell lines has been previously reported.²⁷ The finding that the induction is restricted to one type of the two TNF receptors in stimulated PBL as well as analogous findings in cell lines (Hohmann et al., submitted for publication) indicates that the two TNF receptors are functionally distinct.

DISCUSSION

Most human cells express two distinct TNF receptors simultaneously. The molecular cloning of the 55-kDa receptor^{14,15} and of the 75-kDa receptor (reference 16 and this work) now allows a comparison of the predicted amino acid sequences of both receptors. The

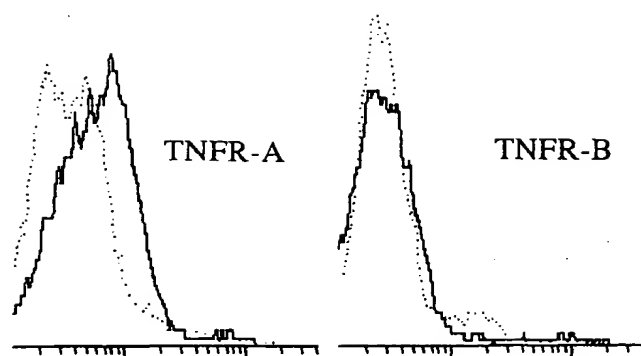


Figure 5. Flow cytometric analysis of 75/65-kDa (TNFR-A) and 55-kDa (TNFR-B) receptor expression on resting (dotted line) and activated (solid line) peripheral blood lymphocytes (PBL).

extracellular regions are found to be highly similar to each other and to those of the NGF receptor and the CDw40 and OX40 antigens. These cell surface molecules thus form a novel gene family. The functional significance of the similarity to the LIM sequence motif²² remains to be established.

Two TNF-inhibitory peptides of human serum and urine have been described and partial amino acid sequences have been reported.²⁸⁻³⁰ One of these inhibitors previously has been recognised as a fragment of the 55-kDa TNF receptor.^{14,15} We now find that the short NH₂-terminal sequence of the second inhibitor³⁰ matches the V⁵-P⁹ peptide sequence of the 75-kDa TNF receptor (Fig. 1). The Northern blot analysis of cell lines (Fig. 4) reveals a single 75-kDa TNF receptor mRNA species of about 4 kb and provides no evidence for a second message which might encode this inhibitor; analogous conclusions are valid for the other inhibitor.¹⁴ Both of these TNF inhibitory peptides therefore are NH₂-terminally truncated, soluble fragments, presumably of the extracellular regions of the two TNF receptors, and therefore are most likely the products of posttranslational processing of the receptor.

The predicted amino acid sequences of the intracellular regions of the two TNF receptors are unrelated and, furthermore, no similarities to other known mammalian sequences were discovered. It might be concluded that the different intracellular domains transmit distinct signals upon TNF binding to the receptors. However, we cannot presently exclude the possibility that the intracellular regions have no role in signal transduction. A model analogous to that of the interleukin 6 (IL 6) receptor might be considered, where the complex of IL 6 and IL 6 receptor can interact extracellularly with a non-ligand-binding membrane glycoprotein, thus providing the IL 6 signal,³¹ both TNF receptors might then address the same signal transducing element. However, in view of the independently regulated expression documented at least in T-cell activation

(Fig. 5) it appears more likely that the two TNF receptors are functionally distinct.

MATERIALS AND METHODS

Cells and Flow Cytometry

The cell lines HL60 (ATCC CCL 240), HEp2 (ATCC CCL 23), Raji (ATCC CCL86) and COS-1 (ATCC CRL 1650) were grown in RPMI 1640 or Dulbecco's modified Eagle's medium supplemented with 10% inactivated horse or fetal calf serum. Human PBL from a Ficoll gradient were cultured in RPMI 1640, 10% fetal calf serum with or without 2 mg/mL phytohemagglutinin (Wellcome). Cells were stained with biotinylated utr-1 (anti-75-kDa receptor) or htr-9 (anti-55-kDa receptor) antibodies followed by streptavidin-phycoerythrin and analysed on a FACScan flowcytometer.

Reagents

Recombinant human TNF- α purified from *Escherichia coli* was a gift from W. Hunziker, E. Hochuli, and B. Wipf (Hoffmann-LaRoche LTD, Basel). TNF- α was radioiodinated with Na¹²⁵I (IMS30, Amersham) and Iodo-Gen (Pierce) to 0.3×10^8 - 1.0×10^8 cpm/ μ g as described.³²

cDNA Cloning and Northern Analysis

The 75-kDa and 65-kDa TNF receptors were purified from HL60 cells, and tryptic digests and gas phase sequencing were performed as reported elsewhere.¹² A DNA fragment was prepared from the peptide sequence Q⁴¹²-L⁴²⁸ by PCR on human genomic DNA using 2 low-stringency annealing cycles (95°C 7 min / to 37°C in 2 min / 37°C 1 min / to 72°C in 2.5 min / 72°C 1.5 min / to 95°C in 1 min / 95°C 1 min / to 37°C in 2 min) followed by 38 standard cycles (95°C 1 min / 55°C 2 min / 72°C 2 min); the forward and reverse PCR primers were ctcgaattcCARCTNGARACNCC and ctcgaattcNARNGGYTTYTCYTC, respectively. The DNA band of predicted size from a polyacrylamide gel of the PCR product was recloned, sequenced, and found to encode the Q⁴¹²-L⁴²⁸ peptide. A 48-mer oligonucleotide derived from this DNA was used as a probe to screen cDNA libraries. Several overlapping clones were identified in a human placenta cDNA library in λ gt11 (Clontech) and in a HL60 cDNA library in λ gt11 that was prepared with the use of cDNA synthesis and cloning kits (Amersham). All recloning and nucleotide sequencing was by standard protocols.³³ For Northern analysis, 12 μ g aliquots of Raji-, HL60-, or HEp2-cell total RNA were electrophoresed through an agarose gel containing formaldehyde. RNA was transferred to a Zeta Probe (BioRad) filter, and hybridized to actin, 55-kDa receptor (full length), and 75-kDa receptor (170-bp 5'-fragment) cDNA probes as indicated.

Expression and TNF Binding in COS Cell Transfectants

The cDNA shown in Fig. 1, truncated at the 3'-end was cloned into a pLJ268 vector (gift of B. Cullen²⁵) containing the IL 2 receptor signal sequence under the control of the RSV long terminal repeat promoter and polyadenylation signals

derived from the rat preproinsulin II genomic gene. DNA was transiently transfected into COS-1 cells with DEAE dextran following standard protocols.³³ Specific ¹²⁵I-TNF- α binding on transfectants was measured in the absence and presence of excess unlabeled TNF- α after 3 days in culture as previously reported¹⁴ and Scatchard analysis was carried out. Briefly, COS-1 cells were detached with EDTA (GIBCO), washed and incubated with ¹²⁵I-TNF- α for 2 hr at 4°C; cell-bound and free radioactivity was then counted. Aliquots of transfected cells were lysed by 1.0% Triton X-100. The expression of the 75-kDa TNF receptor and of the "55-kDa-type" endogenous COS-1 cell receptor was measured in transfectant cell lysates in a solid phase sandwich assay using the 75-kDa and 55-kDa receptor-specific monoclonal antibodies utr-4 and htr-20, respectively, and with ¹²⁵I-TNF- α in the absence and presence of unlabeled TNF- α . The relative receptor expression in the cell lysate in Table 1 is defined as the quotient of specific 75-kDa and 55-kDa TNF receptor ¹²⁵I-TNF- α binding measured in the two sandwich assays. Controls 1 and 2 refer to parallel transfectants in which constructs where the cDNA was ligated into the expression vector in a false reading frame were used.

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Complement

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The term "complement" is used to include a complex group of interacting blood proteins and glycoproteins found in all vertebrates. These proteins have as their primary functions the production and regulation of inflammation, the opsonization of foreign materials for phagocytosis, and the mediation of direct cytotoxicity against various cells and microorganisms. The first evidence for the existence of such a system arose in the late 19th century during studies of the mechanism of host defense against invading bacteria and studies of the mechanism of destruction of foreign or mismatched transfused cells (1-3). These studies demonstrated that individuals are ca-

pable of responding to invading microorganisms or to injected foreign cells by the production of antibody. Such antibodies are able *in vitro* to agglutinate the organisms or foreign cells used in the immunization but are unable to mediate cell death. It was discovered that the addition of fresh serum to a mixture containing specific antibody and the microorganism or immunizing cell often led to cell death. The importance of this property of fresh serum was quickly recognized, and a series of investigations was begun to define the biochemical and biologic basis of the phenomenon of cell lysis.

Work over the next several decades demonstrated the

complex nature of the lysis phenomenon. It quickly became clear that the lytic material, present in all fresh sera, was not a simple substance but could be separated into several principles by even the crude chemical techniques then available. For example, dialysis of the serum against water separated the lytic material into a precipitable euglobulin fraction and a soluble pseudoglobulin fraction. Neither fraction had lytic activity when tested, but mixing the fractions restored this activity. The materials that acted together to produce this cytotoxic response were collectively termed alexin by Bordet, and first "addiment," and later complement by Ehrlich. Although some early workers doubted that the reactions leading to lysis followed simple chemical rules, it was soon recognized that this was indeed the case and experimental systems were established to permit detailed study of these biochemical events. Many of the experimental models established at the turn of the century are still in use today and it is valuable to consider a few of them in some detail.

Much of the early work was directed toward establishing an *in vitro* system designed to allow for analysis of each of the steps involved in complement-mediated cell death. Erythrocytes from many species were screened to determine which were the most easily lysed by antibody and complement. Sheep erythrocytes proved to be particularly useful since, when sensitized with antibody, they were highly susceptible to the lytic action of complement. It was also discovered that sheep erythrocytes have on their surface a potent lipopolysaccharide antigen (termed Forssman antigen) (4) and that high titered antibody to this antigen could be prepared conveniently in the rabbit. In general, sera from all mammals could be used as a complement source, but the degree of lysis varied greatly among different species. It was found that fresh guinea pig serum was the most potent lytic serum easily available. A test system utilizing sheep erythrocytes sensitized with rabbit antibody for studies of lysis in fresh guinea pig serum was commonly employed. With time, it became possible to create specific intermediates bearing various complement components on the surface of the antibody-coated sheep erythrocytes and to study the interaction of each newly defined complement protein with the appropriate cellular intermediate in the complement sequence. Early efforts focused almost exclusively on the events that occur in the lysis of the antibody-coated sheep red cell. Such events now comprise the classical complement pathway. In more recent years it has become clear that another closely related series of proteins is often involved in the lysis of bacteria with or without the intervention of antibody. These bacteriolytic proteins make up the alternative complement pathway. The two pathways of activation converge at the step of C3 activation and engage the later lytic components in the complement cascade (Fig. 1). The classical pathway, in general, is initiated by the formation of an antigen-antibody complex. Recognition of the antigen-antibody complex by the proteins of the classical pathway leads to sequential formation of enzymes with serine protease activity. These cleave and activate C3. The proteins of the alternative pathway mediate this same end result, albeit with slower kinetics of activation. Cleaved C3 (C3b) interacts with the C3-cleav-

ing enzymes of either the classical or alternative pathways and alters their substrate specificities such that they are able to cleave C5. Cleaved C5 (C5b) then interacts with the remaining numbered components, C6, C7, C8, and C9, and these five terminal complement components, acting in concert, mediate cell lysis. This general scheme of complement activation is presented in Fig. 1.

The complement system is essentially entirely conserved throughout the mammalian species that have been studied, and the proteins are often (but not always) interchangeable across species. The system is by far best characterized in the human and guinea pig, although much data on the murine proteins have evolved recently through molecular techniques.

TERMINOLOGY

The nine proteins of the classical pathway are designated by an uppercase letter C followed by a number. The numbers generally follow the order of action of the components, with the exception of C4, which acts before C2 and C3. Components acting solely in the alternative pathway are designated by letters. Regulatory proteins are designated by a descriptive title (e.g., C4 binding protein) or, in the case of those proteins closely associated with the alternative pathway, a letter (e.g., factor H). Single components or multicomponent complexes that have enzymatic activity are designated by a bar over the component(s) in question (e.g., $\overline{\text{C1r}_2}$). Molecules that have lost activity through chemical denaturation or by the action of a control protein are usually designated by a prefix lower case i (e.g., iC3). Fragments or subunits of the various components are designated by a lowercase letter suffix (e.g., C3b).

THE CLASSICAL COMPLEMENT PATHWAY

The Role of Immunoglobulin

Activation of the classical pathway is initiated by the binding of C1, the first component in the cascade, to an antigen-antibody complex and the subsequent activation of the antibody-bound C1 (5,6). The steps have been examined in considerable detail. Not all classes of antibody are capable of binding C1 to initiate the classical pathway. IgG and IgM antibodies have this ability, but IgE, IgD, and IgA antibodies do not (5). Studies in a number of test systems have demonstrated that a single molecule of IgM bound to a particulate antigen is capable of binding one molecule of C1, a complex zymogen protease (7). However, the processes of antibody binding of C1 and the activation of C1 to a protease capable of cleaving C4 and C2 are not equivalent (6). To mediate C1 activation, the IgM antibody molecule must engage the antigen by more than one of its Fab arms. This was inferred from studies of the binding and activation of C1 on anti-hapten IgM sensitized sheep erythrocytes coated with various den-

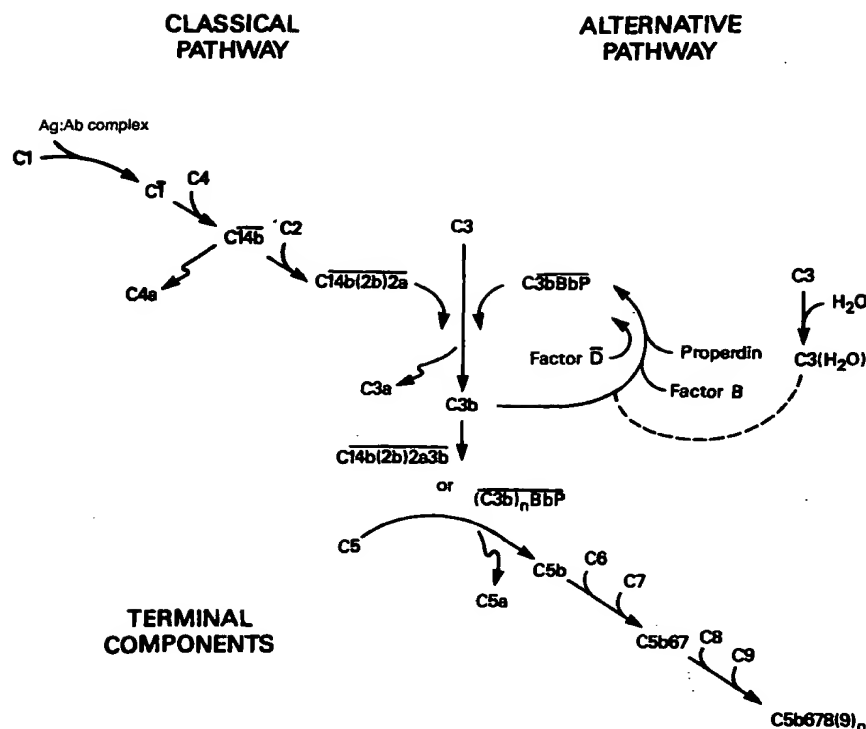


FIG. 1. A schematic diagram of complement activation. Regulatory proteins, side reactions, and inactive fragments have been omitted for clarity. Enzymatically active species are designated by overbars.

sities of a defined hapten, and by analysis of C1 activation by idiotype anti-idiotype immune complexes containing IgM (8,9). The finding that multiple variable region sites must be bound to an antigenic surface for C1 activation suggests that binding facilitates a conformational change in the antibody that promotes C1 activation.

On the other hand, in most of the model systems studied, two IgG molecules side by side (a doublet) are required for C1 binding and activation (10). In molecular terms, the requirement for an IgG doublet greatly reduces the efficiency of IgG as compared to IgM in inducing classical pathway activation. In most systems that examine the lysis of target particles, hundreds or thousands of IgG molecules must be supplied before, by chance, two molecules come to lie sufficiently close together on a surface to produce a doublet. If the distribution of antigen molecules on a surface precludes the possibility of two IgG molecules coming to lie sufficiently close together, the IgG may not activate complement at all. In fact, it has been shown that IgA, under some circumstances, blocks complement activation by IgG antibody by inhibiting the formation of doublets (11). Not all IgG subclasses are capable of activating the classical pathway. Human IgG1, IgG2, and IgG3 are all activators of the classical pathway, but IgG4 is not. In mouse systems, $\gamma 2a$, $\gamma 2b$, and $\gamma 3$ activate the classical complement pathway, and in guinea pig, $\gamma 2$ antibodies activate the classical pathway.

It is not known whether the binding of IgG antibody to its substrate causes a structural change in the antibody that induces increased affinity for C1. Binding of C1 to surface-bound IgG doublets may simply be a consequence of cooperative binding effects. It has been shown that IgG monomer will interact with native C1, albeit weakly (12).

A cluster of closely associated IgG molecules may allow for multiple points of C1 attachment, thus stabilizing the IgG-C1 complex. Binding of C1 proceeds via its attachment to the CH₂ domain of the Fc portion of the IgG molecule (13,14).

Recent data suggest that successful activation of C1 requires multiple points of contact between C1 and the activator. In the case of IgG it has been suggested that not only must the CH₂ domain bind C1 but there must be a second point of contact with the CH₁ domain for activation to occur (15). In keeping with this, it is also suggested that a rather narrow range of angles between the Fab arms of IgG are optimal for C1 activation (8). Accordingly, both simple spatial clustering and structural rearrangements induced by antigen binding may have a role in C1 activation by IgG. Recently, a C1-binding motif consisting of a trio of charged amino acid residues has been identified in the CH₂ domain of murine IgG. These residues are highly conserved in most mammalian IgGs examined and appear to be necessary, but not sufficient, for efficient C1 binding (16).

C1

C1 exists in serum as a three-subunit macromolecule with the subunits held together in the presence of ionic calcium (7,17). C1q is the subunit that binds to an antigen-antibody complex via the antibody CH₂ domain; it has a molecular weight (MW) of about 400,000 and is composed of 18 chains: three chain types termed A, B, and C with six copies of each per molecule. The protein has a central

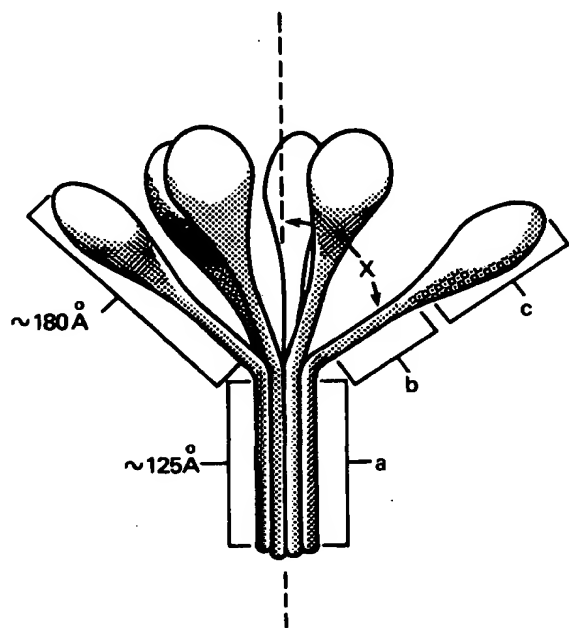


FIG. 2. A conceptualization of C1q. The central core is designated by (a) and the collagen-like fibrillar arms by (b). The globular heads which bind to immunoglobulins are designated by (c). Angle X ranges from 20° to 80°.

core and six radiating arms, each of which ends in a pod-like globular structure (Fig. 2). The amino terminal end of each arm has a triple-helix, collagen-like structure and can be cleaved by bacterial collagenases. These regions are rich in glycine, hydroxylysine, and hydroxyproline. Binding of C1q to the CH₂ domain of the antibody occurs through the podlike carboxyl terminus at the end of each arm. Potentially, each of the six C1q arms can bind to one CH₂ domain; it is assumed that multiple C1q-antibody interactions are required for firm binding (see previous discussion). C1q is found in serum in association with two additional C1 subunits: C1r and C1s. C1r and C1s both have molecular weights of about 85,000 and are single-chain proenzymatic forms of serine proteases. Current models envision the two C1r molecules and the two C1s molecules arranged linearly with the two C1r molecules in contact in the center of the claim and the two C1s molecules at the ends (18). When C1 is incubated in ethylene diamine tetraacetate (EDTA)-containing buffers to remove calcium, the molecule dissociates. The two chains of C1r molecules remain associated and the two C1s chains are free in solution. C1q remains intact but loses its C1r and C1s.

The C1r₂-C1s₂ tetramer is believed to associate with the collagenous regions of the C1q molecule when calcium is present (18). Following binding of the intact macromolecular zymogen form of C1 to an antigen-antibody complex, the C1 undergoes enzymatic activation to become an active serine protease. Activation is associated with cleavage of each of the C1r chains and each of the C1s chains into a heavy chain of 57,000 and light chain

of 28,000 daltons. The enzymatic sites reside in each of the smaller subunits in both cases (19).

In *in vitro* studies, activated C1r is capable of cleaving and activating C1s; activated C1s has broader enzymatic specificity and is the enzyme responsible for the cleavage of both C4 and C2. The mechanism by which the binding of C1q leads to activation of C1r and C1s is unknown. Some have suggested that this involves an intramolecular rearrangement within the intact C1 molecule. The mechanism of C1r cleavage of C1s is also unknown. Although some investigators have suggested that activated C1r cleaves the C1s chains within a single macromolecular C1 molecule, others have hypothesized that C1r cleavage of C1s is actually an intermolecular event requiring the proper alignment of two C1 molecules on an activating surface.

C4

The binding and activation of C1 leads to the generation of an enzyme capable of coordinating with and cleaving the second protein in the cascade, C4. C4 is composed of three disulfide-linked chains termed α , β , and γ with MWs of 93,000, 78,000, and 33,000, respectively. The protein is synthesized as a single-chain precursor (proC4) and the three-chain structure is formed as a post-translational event (20,21).

On interaction with C1s, the C4 α chain is cleaved with release of a small fragment, C4a (9,000 daltons), from its amino terminus. C4a is discussed in detail in the section on anaphylatoxins. The larger fragment, C4b, contains the modified α chain (α'), β , and γ and continues the complement cascade. The binding of C4b to a surface, unlike the binding of C1, proceeds via formation of a covalent ester or amide bond and is apparently highly analogous to C3b binding (see later discussion) (22). An antibody site with a bound, active C1 will cleave multiple C4 molecules, and a cluster of C4 molecules will bind to the region surrounding the antibody-C1 site. This represents an amplification step in classical pathway activation since a single C1-fixing site leads to the activation of multiple C4 molecules. Not all deposited C4 molecules are equally active hemolytically (23). Those which bind to, or close to, the antibody-C1 complex will continue the complement cascade. Bound C4b appears to protect adjacent C1 molecules from the action of C1 inhibitor (24). This serves to promote complement activation at the site of an immune complex and limits the effect of nonspecific activation of C1. The binding of C4b to certain targets may have an effect on biologic activity. For example, certain viruses may be neutralized by the deposition of multiple C4 molecules on their surface, preventing their binding to a suitable host cell (25).

C2

The third protein in the antigen-antibody recognition steps of the classical pathway is C2. This molecule con-

sists of a single peptide chain of 95,000 daltons. In the presence of Mg^{2+} ion, C2 binds to C4b and is cleaved by adjacent $C1s$. Two fragments are formed. A small fragment (C2b, MW 30,000) is cleaved from the molecule, and the larger fragment C2a remains associated with C4b to continue the cascade (26). The complex of C2a and C4b is endowed with new enzymatic activity: the ability to coordinate with and to cleave C3. The active enzymatic site, again a serine protease, resides on the C2a portion of the molecule. Some data suggest that the C2b remains as part of the complex, acting as the C4b-binding site (27). The C4b in the complex binds the C3 molecule and makes it accessible to C2a cleavage. The C4b2a complex, termed the classical pathway C3 convertase, is labile and undergoes decay with physical release of the C2a as an enzymatically inactive fragment. The C4b left behind on the antigenic surface can bind another C2 molecule, which on cleavage by $C1$ will again form the C3 convertase enzyme.

REGULATION OF THE CLASSICAL PATHWAY

C1 Inhibitor

The C1 inhibitor (C1INH) is a single-chain serum glycoprotein of 105,000 daltons with an unusually high carbohydrate content (about 35 to 40%) (28). The protein functions by combining in 1:1 stoichiometry with the active site on each activated $C1r$ and $C1s$ to destroy its protease activity. Since there are two $C1r$ protease sites and two $C1s$ protease sites per $C1$ molecule, one molecule of activated C1 can, in theory, react with four molecules of C1-INH. Binding of C1-INH to activated $C1$ induces disassembly of the C1 molecule with release of two molecules of a $C1rC1s$ (C1INH)₂ complex (29,30). The C1q is presumably left behind on the activating surface where it may interact with additional plasma C1r and C1s or with specific C1q receptors on a variety of cells (see section on receptors). C1-INH chemistry has been examined in considerable detail. It is a member of the family of proteins termed serpins (serine protease inhibitors) and acts by presenting a bait sequence to the enzyme to be inhibited. In the case of the C1-INH, this bait sequence contains a critical arginine at position 444 (31). The enzyme to be inhibited cleaves the C1-INH at the active-site arginine into two fragments of 96,000 and 9,000 MW. Upon cleavage, a reactive site is exposed in the 96,000-dalton fragment which binds to the active site on the enzyme to be inhibited, forming a stable complex that is resistant to boiling in SDS (28). C1-INH is also reported to interact with C1 before its activation. This interaction appears to inhibit C1 activation by spontaneous autocatalysis or non-immune activators but not by antigen-antibody complexes (32). Interestingly, binding of C1-INH to $C1r$ leads to loss of detectable C1r antigen using most anti-C1r antisera. Thus disappearance of antigenically detectable C1r, along with the appearance of cleaved C1r and C1s chains on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), allows one to follow the kinetics of C1 activation and inhibition (33).

C4b Regulation and C4-Binding Protein

The activity of C4b is under the control of a series of membrane-bound and fluid-phase proteins. In this section we describe interactions that can occur in the fluid phase. A separate section discusses all the membrane-bound complement regulators.

C4-binding protein (C4BP) is an interesting molecule of approximately 570,000 daltons. The molecule consists of seven identical chains that are associated by disulfide bonds at their carboxy termini, forming a central "core" (34). The amino terminal portions of the seven chains form tentacular arms which radiate from the core and bear the C4b binding domains, which are homologous to the binding domains of a broad family of proteins that interact with C3b and C4b (see later discussion). C4BP binds to C4b (not C4) and exerts two distinct regulatory actions. The intrinsic dissociation rate of the C4b2a complex is increased, thus shortening the survival of any given classical C3 convertase enzyme (35). In addition, C4b bound by C4BP becomes susceptible to proteolysis by factor I, also known as C3b/C4b inactivator. Factor I cleaves the α' chain of C4b in two places, releasing a four-chain complex termed C4c and leaving a small portion, C4d, covalently bound to the original acceptor surface (36). C4d can no longer support complement convertase activity. Factor I cleavage of C4b on surfaces can proceed without C4BP, albeit slowly, but C4BP is requisite in the fluid phase.

THE ALTERNATIVE PATHWAY OF COMPLEMENT ACTIVATION

History

An alternative pathway for complement activation was described in 1954 by Louis Pillemer and his co-workers at Case Western Reserve University during their investigations of the inactivation of C3 and late-acting complement components by yeast cell walls. They demonstrated that an insoluble yeast cell wall preparation, zymosan, could completely consume C3 during a 37°C incubation in serum without affecting C1, C4, or C2 titers (37). This inactivation had the characteristics of an enzymatic reaction and required factors that could be removed from serum by preincubation with zymosan at 17°C. These factors differed from antibodies in that their absorption from serum required magnesium ions, temperatures above 10°C, a pH of 6.5 to 8.2, and low ionic strength. This led to the suggestion that consumption of C3 occurred by enzymatic activation via a new pathway which was termed the properdin system. Moreover, it was demonstrated that this system played an important role in the serum bactericidal reaction, in viral neutralization, and in the acid lysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria. Unfortunately, subsequent recognition that natural antibody to zymosan is present in normal serum led to the belief that Pillemer's data reflected only the activation of the classical pathway

by preexisting antibody (38). Thus the fundamental discoveries of Pillemer and his colleagues were largely ignored until the alternative pathway was "rediscovered" a decade later.

There are six normal serum proteins thought to be important in the initiation and control of alternative pathway activation. These are factor B, factor D, properdin, factor H ($\beta 1H$), factor I (C3b/C4b inactivator), and C3 itself (39).

Factor D

Factor D is a 25,000-dalton, single-chain glycoprotein, with γ -globulin electrophoretic mobility when purified but α -globulin electrophoretic mobility in serum (40). It is a trace protein in serum (1 to 2 $\mu\text{g/ml}$), and its low concentration may make it rate limiting in assembly of the alternative pathway C3 convertase, C3bBb. No evidence of cleavage of factor D has been found and it is presumed to circulate in the active state. It has sequence homology with other serine proteases but does not hydrolyze synthetic esters and is relatively resistant to the archetypal serine protease inhibitor diisopropylfluorophosphate (DFP) (41). It has no activity on its natural substrate, factor B, until the latter is bound by C3b. Thus it is most likely that factor D is able to express its proteolytic activity only after exposure of a cryptic site on factor B by the C3b interaction.

Factor B

Factor B is a single-chain, 93,000-dalton, β -globulin zymogen serine protease. In the presence of Mg^{2+} , factor B forms a stoichiometric complex with C3b with a molar ratio of 1:1 (42). On cleavage by factor D, two fragments are formed. The smaller 30,000-dalton fragment, Ba, is released, while the larger 63,000-dalton fragment, Bb, remains associated with C3b. The latter fragment contains the protease activity (43). The complex C3bBb is termed the alternative pathway C3 convertase. This complex is quite labile and Bb dissociates spontaneously from C3b under physiologic conditions. Ba and Bb are reported to have opposing regulatory effects on B lymphocyte function and Bb is reported to augment the spreading of phagocytic cells on a surface.

Properdin

Properdin (from the Latin *perdere*, to destroy), the protein through which the alternative pathway was discovered, is a γ -globulin of approximately 143,000 to 156,000 daltons, consisting of three apparently identical subunits held together noncovalently (44). Its serum concentration is about 25 $\mu\text{g/ml}$. There are two forms of properdin, native (nP) and activated (P), which apparently differ from each other only by a small conformational change (45). Native properdin can bind to the assembled alternative

pathway C3 convertase (C3bBb) but cannot bind to C3b alone. Its function in this circumstance is to reduce the rate of decay of the convertase and thus promote alternative pathway activation. Activated properdin can, in addition, bind to C3b on particles or in the fluid phase in the absence of factor B and then promote the assembly of C3bBb. Factors regulating the conversion of nP to P are largely unknown, and some authors have questioned whether two distinct forms truly exist. Spontaneous conversion of nP to P in purified protein preparations has been observed to occur, and P is the form of the molecule eluted from alternative pathway activators after incubation in serum. On the other hand, P does not revert to nP spontaneously but is reported to do so after incubation with the denaturant guanidine.

Factor H

Factor H (formerly known as $\beta 1H$) is a 150,000-dalton, single-chain peptide of β electrophoretic mobility. Its serum concentration is about 500 $\mu\text{g/ml}$ (46). Gel filtration studies indicate that factor H may circulate as a dimer under physiologic conditions. Unlike the previously described proteins, factors B and D and properdin, which are important in the assembly of the alternative pathway C3 convertase, factor H functions to downregulate C3-cleaving convertase activity. This is accomplished by competition with both B and Bb for C3b binding, thereby inhibiting convertase formation and accelerating decay of existing convertase complexes (47). Factor H is also a necessary cofactor for the inactivation of C3b by factor I (48). The binding of H to C3b does not require cations but is enhanced by low ionic strength. Factor H can bind to C3b on surfaces or in the fluid phase, although with varying affinities depending on the chemical nature of the environment in which the C3b is found. As explained later, this varying affinity of H for C3b may be the most important determinant of whether or not a particle will activate the alternative pathway.

Factor I (C3b/C4b Inactivator)

Factor I is a 90,000-dalton, β -globulin glycoprotein composed of two chains of 50,000 and 40,000 daltons held together by disulfide bonds (49). It demonstrates proteolytic activity toward two substrate molecules: C3b and C4b. In the presence of the appropriate cofactors, factor I effects two sequential cleavages of C4b and three cleavages of C3b (50). These result in defined breakdown products with altered activity (see later discussion). The active site is on the 40,000-dalton light chain and is not inhibitable by DFP, soybean trypsin inhibitor, tosyl-L-lysine chloromethyl ketone (TLCK), benzamide, or phenylmethyl sulfonyl fluoride (PMSF). Nonetheless, sequence analysis has demonstrated a high degree of homology between factor I and known serine proteases. Thus the activity of this enzyme is probably controlled by a requirement for configurational changes in its substrates (C3b or

C4b) mediated by essential cofactors (factor H or C4-binding protein), before exposure of the site for enzymatic cleavage. This is analogous to the mechanism of enzymatic activity of factor D on factor B.

C3

C3, because of its central role in both classical and alternative pathway activation and because of its major role in the host defense process, has been the focal point of much research on complement activation. Human C3 is a 195,000-dalton glycoprotein of β electrophoretic mobility. It has two chains, one of 120,000 daltons (α chain) and the other of 75,000 daltons (β -chain) with carbohydrate present on both chains (51). Serum concentration is 1 to 2 mg/ml. C3, like C4, is synthesized as a single-chain precursor molecule (proC3) in which the β chain occupies the amino terminus. Excision of a series of four arginine residues yields the mature molecule.

Within the α chain resides an unusual thioester bond between a cysteinyl residue and a glutamic acid residue, separated by two intervening amino acids. This unstable bond, thought to be buried in a hydrophobic pocket, is responsible for the covalent binding reactions of C3 (as well as C4 and α_2 -macroglobulin, which share this feature) (52).

When C3 is cleaved by either the classical pathway C3 convertase (C4b2a) or the alternative pathway C3 convertase (C3bBb), the α chain is divided into two unequal fragments, the larger of which remains covalently linked via disulfide bonds to the β chain. This molecule, designated C3b, has a MW of 185,000 daltons (53). The smaller fragment, C3a, represents the amino terminal 77 amino acids of the α chain and has diverse effects on cells with receptors for this peptide, including lymphocytes, mast cells, and endothelial cells (see later discussion). On cleavage of C3 to C3b, the molecule undergoes a complex rearrangement of tertiary structure, which exposes the internal thioester bond in the α chain (52,54). On exposure, the thioester bond can be broken by reaction with appropriate aldehydes, carboxyl groups, nitrogen nucleophiles, or by water itself. The result is a new covalent bond between C3b and an electron-donating group. On particles such as erythrocytes or zymosan, the formation of ester bonds seems to be favored (55), whereas proteinaceous immune complexes form amide, as well as ester, bonds with C3b. Since water can hydrolyze the thioester and is generally present in vast molar excess over all other potential electron donors, the process of C3b binding to surfaces or soluble immune complexes is inefficient. In general, many molecules of C3b will incorporate H_2O into the reactive bond for each molecule that is able to bond covalently to the complement-activating surface. Nonetheless, recent experiments in which C3 was cleaved to C3b in the presence of small molecules, such as amino acids and simple sugars, have demonstrated that some of these are preferential acceptors of the C3b molecule. In particular, glycerol, threonine, and raffinose are highly efficient at forming covalent bonds

with C3b even in the presence of a large excess of H_2O (56). Immunoglobulin G also appears to be an efficient C3 acceptor (see later discussion).

C3b, after formation by either the classical or alternative pathway C3 convertase, is susceptible to further cleavage, which proceeds in several steps (see Fig. 3) (57,58). The first cleavage is made by factor I with H as an essential cofactor for fluid-phase cleavage and an accelerator for cleavage on surfaces. A second factor I-mediated α -chain cleavage occurs in rapid succession with the release of a 3,000-dalton fragment. The resultant three-chain molecule is termed iC3b. It can no longer function in the alternative pathway C3 convertase (or the C5 convertase of either pathway). The iC3b molecule has 68,000- and 43,000-dalton chains derived from the α' chain of C3b and the unchanged 75,000-dalton β chain of the original molecule. Surface-bound iC3b undergoes a third cleavage at a site within the 68,000-dalton chain, which releases a three-chain, 145,000-dalton molecule termed C3c and leaves a 41,000-dalton fragment termed C3dg bound to the surface. This cleavage is carried out by factor I, but factor H does not appear to be a physiologically relevant cofactor (58). Instead the cell membrane C3b receptor, called CR1, appears to serve as cofactor for this step (see later discussion). A variety of enzymes, including trypsin, plasmin, and neutrophil elastase, can remove a 10,000-dalton amino terminal portion from C3dg to yield C3d. These same enzymes can generate C3d directly from iC3b. The breakdown of iC3b in the fluid phase appears to follow the same steps but proceeds very much more slowly and is less well characterized.

ALTERNATIVE PATHWAY ACTIVATION AND CONTROL

When C3b is generated in the course of classical pathway activation, it may serve as an initiator of the alternative pathway by providing a binding site for factor B. The binding requires Mg^{2+} . Binding to C3b is presumed to expose a cryptic cleavage site in the factor B molecule, which is the substrate for the protease, factor D. Ba is released, and the resulting bimolecular complex, C3bBb, acts as the C3 convertase of the alternative pathway (59). The active enzymatic site is on Bb, but C3b is essential. C3b in the alternate pathway convertase is able to bind additional native C3 and in so doing makes the cleavage site in the C3 molecule available to the enzymatic activity of Bb. In this way, C3b in the alternative pathway C3 convertase is analogous to C4b of the classical pathway C3 convertase and Bb is analogous to C2a. Factor D is not incorporated into the C3 convertase and may be reutilized. Enzymatic activity is controlled by several mechanisms. Factor H may displace factor Bb from the alternative pathway C3 convertase and also functions as a cofactor for factor I cleavage of C3b, terminating its ability to function in the convertase (60). In addition, the complex enzyme C3bBb has a tendency to dissociate even in the absence of regulatory proteins, with a $T_{1/2}$ of about 5 min at 30°C. Properdin counterbalances these controls

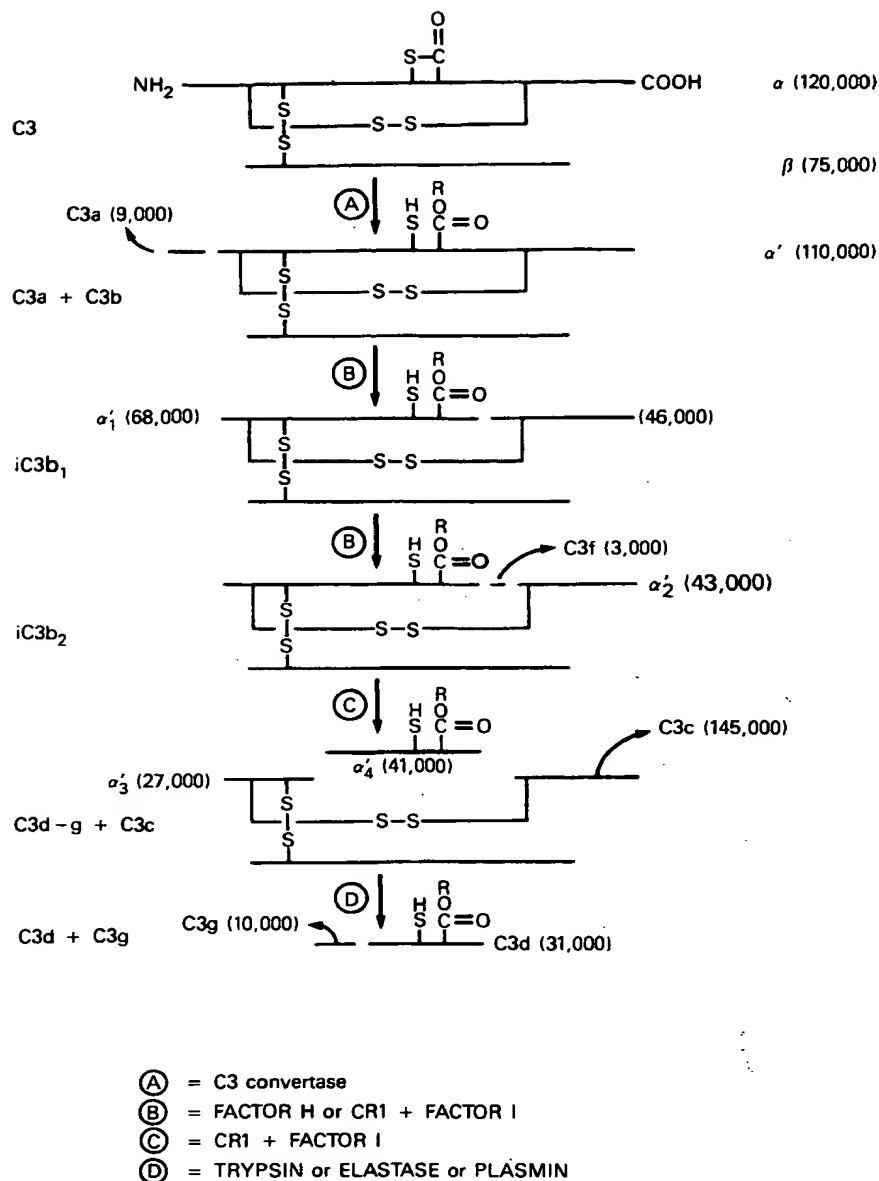


FIG. 3. C3 cleavage and the subsequent stepwise breakdown of C3b. Fragment molecular weights and enzymes responsible for the various cleavages are designated. Recent studies suggest that gp45-70 (membrane cofactor protein) is also a relevant cofactor for step B.

by decreasing the rate of the dissociation of C3bBb and therefore stabilizes enzymatic activity (59). Factors H and I are absolutely required to prevent spontaneous fluid-phase assembly of the alternative pathway C3 convertase and the subsequent consumption of alternative pathway components through unregulated activity of factor D and C3bBb. This has been shown *in vitro* using the purified proteins of the alternative pathway and has also been confirmed *in vivo*, since patients genetically deficient in factor I or H have very low C3 levels and circulating C3 fragments (61). When additional C3b is formed by the action of C3bBb, some portion of the new C3b may join the initial complex to form (C3b)₂Bb. The second C3b provides a binding site for C5 and allows the new complex enzyme to function as the alternative pathway C5 convertase. Kinoshita et al. have very recently demonstrated that the

second C3b responsible for C5-binding activity is actually covalently attached to the initial membrane-bound C3b residue.

Whenever C3b is created by classical complement pathway activation, it may serve as the nidus for formation of an alternative pathway C3 convertase. The alternative pathway convertase can deposit more C3b on the target surface, which can in turn form another alternative pathway convertase site. In this situation, the alternative pathway serves as an amplification mechanism for complement activation and is often referred to as the "amplification pathway."

The initial event of alternative pathway activation in the absence of classic pathway activation is less clear. The most likely sequence of events has been outlined by Pangburn and Muller-Eberhard (59,62). They showed that

the thioester bond in native C3 is not completely stable but subject to slow hydrolysis even in the absence of cleavage of the C3 molecule to C3a and C3b. C3 bearing a hydrolyzed thioester is termed C3(H₂O) and has a number of properties of C3b, including the ability to initiate the alternative pathway by interacting with factor B, the ability to interact with factors H and I, and the capacity to serve as a ligand for cellular C3b receptors (63). Spontaneous hydrolysis of C3 with formation of an alternative pathway C3 convertase leads to a continuous low-level cleavage of C3 in serum called C3 "tickover." This baseline C3 turnover occurs at a slow rate because it is controlled by factors H and I. In the presence of alternative pathway-activating surfaces, such as rabbit erythrocytes, zymosan, or lipopolysaccharide, the tickover process is thought to provide the initial C3b, which binds to these surfaces to begin formation of the surface-bound alternative pathway C3 convertase.

Clearly, not all surfaces support the assembly of an alternative pathway C3 convertase whenever C3b by chance is bound to them through serum C3 tickover. This implies that inherent in the activation of the alternative pathway is the ability to discriminate among surfaces to which C3b binds. A further implication, since normal self-antigens do not activate the alternative pathway, is that the alternative pathway represents a primitive mechanism for distinguishing self-antigens from foreign antigens, such as yeast cell walls (zymosan) or bacterial cell walls or lipopolysaccharide. The molecular nature of this discriminatory ability is partially understood. Activators of the alternative pathway provide a "protected site" for C3b binding, that is, one in which the net effect is to favor factor B binding to C3b over factor H binding (59). In this setting, the alternative pathway C3 convertase will form and be able to cleave more C3 without the usual inhibition by factor H. With rare exception, the property of alternative pathway-activating potential correlates with a reduced affinity of surface-bound C3b for factor H. This has best been demonstrated in investigations of the role of cell surface sialic acids in control of alternative pathway activation (64,65). Although sheep erythrocytes do not activate the human alternative pathway, sheep cells from which sialic acid residues have been removed will activate the alternative pathway, and the extent of activation is proportional to the amount of sialic acid removed. When the mechanism for this was investigated, it was discovered that, although C3b molecules on normal and desialated sheep erythrocytes demonstrated equal affinity for factor B, the affinity of C3b for factor H on fully sialated cells was 10- to 20-fold greater than on desialated cells (66). Thus, in this case, cell surface sialic acid, by increasing the binding of H to C3b, prevents the formation of an effective alternative pathway C3 convertase. A similar mechanism for control of alternative pathway activation was demonstrated for heparin that had been linked to zymosan particles. Interestingly, removal of sialic acid from human erythrocytes does not lead to alternative pathway activation. Thus additional control mechanisms are operative on human cells, some of which are discussed subsequently.

Role of Immunoglobulin

No obligate role for antibody exists in the activation of the alternative pathway, but immunoglobulin can exert a strong modulating influence on this system (67). Aggregated human myeloma proteins of the IgG, IgM, IgA, and IgE classes are capable of activating the alternative pathway (68). Soluble and particulate antigen-antibody complexes formed of IgG from several mammalian species and intrinsically nonactivating antigens have also been shown to activate the alternative pathway, and this can be shown to occur without classical pathway participation. Additionally, sensitization with specific IgG significantly augments the rate and/or extent of alternative pathway activation by intrinsically activating surfaces. This effect is independent of the Fc portion of the IgG molecule and has been observed to occur with both F(ab')₂ and Fab fragments.

The mechanisms whereby IgG influences the alternative pathway are several. IgG is an excellent acceptor of nascent C3b, the majority of which binds to a site in the heavy chain and probably within the Fd fragment (69). Thus antibody may enhance the deposition of C3b and the rate of formation of new C3 and C5 convertases by providing additional acceptor sites on a sensitized surface. Alternatively, antibody may function by masking sialic acid residues that would normally inhibit alternative pathway activation, as has been shown using antibodies to the capsule of group B streptococci (70). Finally, data suggest that C3b covalently bound to IgG is less susceptible to inactivation than free C3b or C3b bound to a non-immunoglobulin acceptor protein (71). This appears to be due to a reduced affinity of factor H for C3b bound to IgG. The relevance of this finding to surface-bound C3b remains to be established, but it may in part explain the observed relative resistance of C3b bound to IgG-bearing soluble antigen-antibody complexes to the action of factors H and I (72).

An interesting autoantibody has been shown to influence alternative pathway activation in a manner quite different from those previously discussed. This antibody, termed C3 nephritic factor (C3NeF), was discovered in the sera of patients with membranoproliferative hypocomplementemic glomerulonephritis and was subsequently shown to be present in some individuals with partial lipodystrophy (73). C3NeF is an IgG antibody specific for the alternative pathway C3 convertase, C3bBb. The complex C3bBbC3NeF is highly resistant to factor H-mediated dissociation and is thus capable of promoting unregulated C3 consumption by the alternative pathway.

Agents that Modify Alternative Pathway Activation

There are a number of substances that have proved to be useful modulators of alternative pathway activation in the laboratory. These can be divided into two groups, those that promote C3 cleavage and those that inhibit C3 cleavage.

One of the most widely used of such reagents is a 140,000-dalton protein from cobra venom that is functionally analogous to C3b. This three-chain molecule, termed cobra venom factor (CVF), is derived from the cobra's C3 (74). It can bind factor B, permit its cleavage by D, and form an alternative pathway C3 convertase. Unlike C3b, CVF is not inactivated by H and I and therefore forms an unregulatable C3-cleaving enzyme. The unregulated convertase is able to cleave C3 and factor B in the fluid phase until both proteins are essentially completely consumed from serum. This activity of CVF has been used by investigators to deplete C3 both *in vivo* and *in vitro* and as a way of obtaining C3b. The extent of consumption of terminal complement components following CVF activation of the alternative pathway depends on the source of CVF. Cobra venom factor isolated from the venom of *Naja naja kaoutha* forms not only an unregulated C3 convertase but also an effective C5 convertase. C5 cleavage is followed by consumption of all the terminal components. Cobra venom factor from *Naja haje* venom, on the other hand, is incapable of C5 cleavage; thus its use leads to consumption of B and C3 but leaves terminal component levels unaffected. The molecular mechanism for these differences in CVF molecules has not yet been elucidated.

Other agents that promote alternative pathway activation include suramin, a drug used in the treatment of African trypanosomiasis, and K-76 monocarboxylic acid, a product of the fungus *Stachybotrys complementi* K-76 (75,76). Both inhibit cleavage of C3b by factors H and I, albeit by different mechanisms.

Agents that inhibit alternative pathway activity include heparin and gold sodium thiomalate, both of which appear to increase the affinity of C3b for factor H (77,78). Fluid-phase heparin also appears to be capable of "masking" a portion of surface-bound C3b residues and preventing their interaction with factor B. Complestatin, a product of *Streptomyces lavendulae*, inhibits alternative pathway activation by combining with factor B in a manner that blocks its interaction with C3b. Recently, a macromolecular product of *Aspergillus fumigatus* has been identified which inhibits alternative pathway activation by as yet unclear mechanisms (79). In addition, a membrane glycoprotein encoded by herpes simplex type 1 virus has been shown to modulate both the alternative pathway C3 convertase and C3-C5 interactions (80). This may result in enhanced resistance of the virus to neutralization in serum (81).

THE C1 BYPASS PATHWAY

There is a third pathway of complement activation, discovered by the use of C4-deficient guinea pig serum. In this pathway, although no classical pathway C3 convertase is formed, C1 activation is required for activation of the complement cascade. Therefore this pathway has been called the C1 bypass pathway (82). Although the mechanism for this effect is unknown, one possibility is that activated C1 replaces D (albeit inefficiently) in con-

centrated C4-deficient serum. Initiation of complement activation via this pathway requires a large amount of antibody. The molecular natures of the C3 and C5 convertases in this pathway are unknown. Recently, this pathway has been shown to be responsible for the lysis of *Giardia lamblia* as well as several bacterial species (83).

TERMINAL COMPLEMENT COMPONENTS AND THE LYTC MECHANISM

A detailed understanding of the molecular interactions of the terminal components C5 to C9 has developed over the last two decades. It is known that biologic activity of C5 requires cleavage of the C5 molecule into two fragments, C5a and C5b, by an enzyme derived from the C3 convertase. The C5a fragment has potent independent biologic activity which is considered later. The C5b portion combines sequentially with C6, C7, C8, and C9 to form a macromolecular complex that is capable of damaging biologic and artificial membranes and causing cell lysis by the creation of a hydrophilic transmembrane pore or channel (84,85). In addition, a phenomenon known as reactive lysis has been described, in which the isolated metastable C5b6 complex can be used to initiate formation of the terminal complex on surfaces without a concomitant requirement for earlier components. This reaction has provided a useful tool for identifying potentiators and inhibitors of terminal complex formation (86). The most intensive areas of investigation have focused on the exact molecular composition of the C5b-9 complex, the biochemical nature of attachment of this complex to membranes, and the functional and biochemical characteristics of the lytic complement lesion. After much debate, most workers in the field agree that complement lysis is due to the formation of a stable, hydrophilic transmembrane channel, as predicted by the "doughnut model" of Mayer et al. (85). There is now a general consensus that complement lysis of cells results because the terminal complex creates membrane channels that are large enough to permit exchange of small molecules and ions but too small to permit release of macromolecular cytoplasmic constituents. Due to the Donnan effect, water enters the cells through such channels causing the cell to swell and burst.

A large body of literature now substantiates that during formation of C5b-9, the complex inserts into the lipid bilayer of membranes. Evidence includes the observations that (a) hydrophobic domains are exposed within the forming C5b-9 complex, (b) phospholipids are released from target membranes during complement attack, (c) the attached C5b-9 complex cannot be eluted from membranes by ionic manipulations or aqueous-phase proteases but instead requires detergent for extraction, (d) conductivity changes are detected across planar lipid bilayers attacked by the terminal complex, and (e) constituents of C5b-9 can be labeled by membrane probes that localize exclusively within the hydrophobic core of lipid bilayers.

Initiation of C5b-9 Formation: C5 and the C5 Convertase

Initiation of membrane attack complex (MAC) formation requires cleavage of C5. C5 is a β -globulin glycopro-

tein comprising a 115,000-dalton α -chain disulfide linked to a 75,000-dalton β chain. Considerable sequence and structural similarity exists between C3, C4, and C5, but the latter lacks a thioester and does not form covalent bonds with target surfaces (51). Physiologic cleavage of C5 produces a 185,000-dalton C5b fragment and an 11,000-dalton C5a peptide. In cellular systems, the hemolytically active C5b remains cell associated whereas the smaller C5a fragment is released into the fluid phase. Recent data suggest that release of C5a is accelerated by interactions of C6 and C7 with newly cleaved C5 (87).

C5 is cleaved by enzymes formed during both alternative and classical pathway activation. The C5 convertase enzyme of the classical pathway is the C4b2a3b complex. Enzymatic activity resides in the C2a molecule within the complex. The enzymatic site for C5 cleavage by the alternative pathway convertase, (C3b)₂Bb, is contained within Bb. In both the classical and alternative pathway C3 convertases, physical decay of the enzymatic subunit from the complex results in loss of C5 convertase activity. Cleavage of C5 by either convertase results in identical fragmentation of the molecule. In each convertase, C3b binds native C5, exposing the site of enzymatic cleavage. Current data indicate that C3b covalently attached to C4b is the relevant C5-binding site in the classical pathway enzyme (88). Recent evidence suggests that there are two molecular forms of cell-bound C3b in the alternative pathway C5 convertase. One molecule appears to function like C4b in the classical pathway convertase, and one molecule is analogous to C3b of the classical pathway enzyme. The C3b serving as a C4b analog binds Bb, maintaining it in an enzymatically active configuration. Fluid-phase C3b can also function as a binding site for C5, with subsequent cleavage by a fluid-phase enzyme complex. It is likely that the major requirement for effective cell surface C5 binding and cleavage is a high local concentration of C3b.

There is a linear relationship between the number of cell-bound C5 convertase complexes and subsequent C5 uptake. However, under conditions in which all C5 hemolytic activity is consumed, only a small percentage of C5 binds to the activating surface. Moreover, cell-bound C5b rapidly loses hemolytic activity despite a substantially slower rate of loss of C5 antigen from the cell surface. Thus it appears that cell-bound C5b undergoes a rapid conformational change that renders it hemolytically inactive. The nature of the change is still unclear. As discussed in more detail later, the presence of C6 and C7 increases the uptake of C5 onto membrane surfaces and stabilizes the hemolytic activity of the cell-bound C5b.

Noncomplement-Mediated C5 Cleavage

A large number of noncomplement proteases produce cleavage of C5 to yield biologically active peptides. A detailed review of these reactions is beyond the scope of this chapter but has been well summarized (89). Briefly, trypsin, plasmin, polymorphonuclear leukocyte proteases such as elastase and cathepsin G, macrophage and platelet

proteases, and bacterial enzymes have been shown to cleave native C5 into biologically active peptides. It is now clear that the fragments produced by trypsin cleavage of C5 are not equivalent to the fragments formed when C5 has been cleaved by a C5 convertase. Specifically, C5a is not produced, although a C5a-like biologic activity is generated. The anaphylatoxic and chemotactic activity of trypsin-cleaved C5 resides in fragments that remain disulfide linked to the high-MW, C5b-like polypeptide.

C5b6 and Reactive Lysis

C6, the next molecule in the cascade, is a β_2 -globulin with a molecular weight reported between 105,000 and 128,000 daltons. Its structure is stabilized by numerous intrachain disulfide bonds (90). C5b and C6 can form a stable complex in serum that retains its ability to interact with cell membranes over time. With the addition of C7, C8, and C9, the C5b6 complex can lyse unsensitized erythrocytes and certain other cells in the absence of the antecedent components, a phenomenon known as reactive lysis (86). The conformational alterations in C5b which enable interactions with C6 are very short-lived, and C5 cleavage must occur in the presence of C6 for effective C5b6 complex formation to occur.

The biochemical characteristics of the C5b6 molecule have been defined (91). The complex has an S rate of 10.4 to 11.5, a MW of 328,000 to 330,000, and in its hemolytically active form contains a single molecule each of C5b and C6. The electrophoretic mobility of the isolated complex is indistinguishable from that of C5 (β_1) but faster than that of C6 (β_2). The C5b6 complex expresses a new antigenic determinant (neoantigen) that is not present on either C5 or C6 and is presumably related to conformational changes in the C5b and C6 molecules during complex formation (92).

C7, Formation of C5b67, and Inhibitors of C5b67

C7 has physical characteristics very similar to C6 and exhibits extremely high affinity for the C5b6 complex. It is reported to have 23 to 30% sequence homology with the later-acting components C8 and C9 (93). Attachment of C7 to newly formed C5b6 occurs very rapidly via hydrophobic interactions and results in the formation of a labile fluid-phase complex that aggregates and loses activity if not attached to acceptor surfaces. The C5b67 complex expresses a unique second neoantigen that differs from that of C5b6. This trimolecular complex represents the first product of complement activation to stably insert into the lipid bilayer of target membranes (94). The activity of the complex is very evanescent at physiologic temperatures and membrane insertion is inefficient (less than 1%) unless the target is sensitized with C3b. The presumptive mechanism of the C3b effect is binding of C5b to C3b, thereby promoting C5b67 formation close to the target surface and increasing the likelihood of subsequent hydrophobic interactions (95).

A number of substances have been defined that act on C5b67 reversibly in the fluid phase to prevent attachment to bystander erythrocytes during the short time that the hydrophobic binding site of C5b67 is available. One class of inhibitors includes the low-density lipoproteins and serum S protein, each of which presumably binds to the nascent hydrophobic membrane-binding site on C5b67 and blocks subsequent interaction of the complex with target membranes. A second class of inhibitors includes various polyanions such as heparin, dextran sulfate, and DNA. On the other hand, histones and polycations enhance the lytic activity of C5b67 for bystander erythrocytes. The mechanisms by which these inhibitors and potentiators act are still unclear, but presumably they function by modifying ionic or charge interactions of C5b6 or C5b67 with the target membrane. It has also been suggested that C8 limits the activity of C5b67 by preventing the attachment of the fluid-phase complexes to target membranes. Thus C8 serves a dual role in complement-mediated cytolysis and represents a point of internal regulation in the late steps of the complement attack sequence.

C8

C8 is a three-chain γ -globulin with α and β subunits of 64,000 daltons and a smaller γ chain of 22,000 daltons. The α and β chains differ in sequence despite their identical molecular weights (93,96). The α and γ chains form a disulfide-linked heterodimer which associates noncovalently with β chain in serum, where the complex exists as an equilibrium mixture. A single molecule of C8 can interact via its β chain with each C5b67 complex and this interaction has an extremely high affinity constant similar to that of C7 uptake by C5b6. When C8 binds to C5b67 on a membrane, the C8 γ chain is inserted into the hydrophobic regions of the phospholipid bilayer as assessed by labeling with hydrophobic photoreactive probes and inaccessibility to proteases. Insertion of C8 α chain through the lipid bilayer constitutes a lesion sufficient to initiate erythrocyte lysis, leakage of radiolabeled solutes, and significant increases in membrane conductivity (84). Target lysis by C5b-8 complexes is slow, however, and the hydrophilic lesions which are apparently formed are both small (0.4 to 3 nm) and unstable. Dramatic enhancement of lytic efficiency occurs on addition of the next component, C9.

C9

C9 is a single-chain α -globulin of 71,000 daltons. It is composed of two distinct domains. The smaller, C9a, is rich in acidic residues and hydrophilic. The larger domain of 37,000 daltons, C9b, is enriched for hydrophobic residues, although the deduced amino acid sequence does not include a single extended hydrophobic region similar to those of typical transmembrane proteins (94). Both C9a and C9b contain multiple intrachain disulfides which ap-

parently do not cross domain boundaries. Antibodies elicited by denatured C9b are cross-reactive with C6, C7, and C8 α chain, as well as the pore-forming protein perforin, isolated from cytotoxic lymphocyte granules. Thus these proteins may have similar structures responsible for their hydrophobic interactions (94).

C9 has the capacity to polymerize, and purified C9 can be induced to do so by prolonged 37°C incubation. Polymerization is accompanied by elongation of the molecule, display of a distinctive neoantigen, an increase in surface hydrophobicity, and the appearance of free sulfhydryl groups. These latter participate in interchain disulfide bond formation, to produce C9 dimers. Polymerized (or "poly") C9 forms highly ordered tubular structures. These have a length of approximately 16 nm and an internal diameter of approximately 10 nm. One end bears a thickened annulus, while the other is hydrophobic and readily inserts into phospholipid bilayers. The average molecular weight of poly C9 is 1.1×10^6 daltons, but the complexes are heterogeneous and may contain 11 to 19 C9 monomers, although the more common forms contain 14 to 16 (84,94,97).

CHARACTERISTICS AND COMPOSITION OF THE FLUID-PHASE AND MEMBRANE-BOUND MEMBRANE ATTACK COMPLEX (MAC)

There is still controversy surrounding the details of complement lysis (84,94,97). In most situations the attack mechanism of complement requires the participation of C5b, C6, C7, C8, and C9 as a macromolecular complex with a molar composition of 1C5b:1C6:1C7:1C8:3-6C9. A stable fluid-phase 22.5S complex with a MW of 1.04×10^6 with the electrophoretic mobility of an α -globulin and containing C5b-9 can be demonstrated after incubation of serum with alternative or classical pathway activators. The complex assembled in the fluid phase has no activity but is able to inhibit lysis of EAC₁₋₈ by C9, presumably because the fluid-phase complex, with a molar ratio of 1C5b:1C6:1C7:1C8:3C9, binds additional C9. Such complexes also contain three molecules of an additional protein, S protein (97). S protein circulates as a normal constituent of serum at a concentration of 600 $\mu\text{g/ml}$. This protein binds to the nascent hydrophobic binding site of C5b67 during formation of fluid-phase terminal component complex, thereby destroying its lytic activity and preventing its aggregation.

The physical characteristics of the membrane-bound MAC (C5b-9) have been studied following extraction from erythrocyte membranes by nonionic detergents. The complex within the membrane behaves like an intrinsic or integral membrane protein since it cannot be extracted with high ionic strength buffers or EDTA. This observation provided some of the first evidence that the MAC associated with the membrane through hydrophobic interactions. The dimensions of the extracted complex as determined by electron microscopy suggested the presence of monomeric C5b-9 complexes with an estimated MW of 1×10^6 . However, others have since reported that the

membrane-bound form of the terminal complex is a C5b-9 dimer or consists of monomeric, dimeric, and trimeric forms, and the MAC may in fact have a heterogeneous size distribution.

Analysis of both cell-bound and fluid-phase C5b-9 complexes has suggested that they contain a disulfide-linked dimer of C9. Such C9 dimers may serve an important function in cell lysis by facilitating dimer formation of the entire C5b-9 complex, thus enhancing membrane disruption. However, this hypothesis remains to be proved.

Electron microscopic studies of the lytic complement lesion using the negative stain technique demonstrated that the lesion has the appearance of a doughnut, with an annular rim of 15 nm in diameter and a central, electron-dense region of 10 to 11 nm in diameter raised above the membrane (Fig. 4) (98). More detailed examination of the detergent-extracted complex from erythrocyte membranes has led to a model in which the complex contains a cylindrical stalk of 15 to 16 nm in length constituting the portion of the complex that is embedded into the hy-

drophobic core of the membrane. The annulus or torus of the complex projects above the membrane by at least 10 nm (Fig. 4), has an external diameter of 20 to 25 nm, and an internal diameter of 10 to 11 nm for human complement and 8.5 to 9.5 nm for guinea pig complement. When the isolated complex is reincorporated into lipid vesicles, the cylindrical axis of the complex is oriented perpendicularly to the membrane, and the annulus is located external to the vesicle (Fig. 4).

The interpretation of the electron microscopic appearance of the terminal complex has been modified in light of data developed by Podack and Tschopp (review in ref. 97). Based on the remarkable similarities between poly C9 and extracted MAC formed in C9 excess, these investigators have proposed that the membrane-bound MAC is composed largely of poly C9, with more limited participation of C5b678. Studies with membrane-restricted hydrophobic photoreactive probes are consistent with this view, since addition of C9 to membranes bearing C5b678 appears to reduce the exposure of the C5b678 to

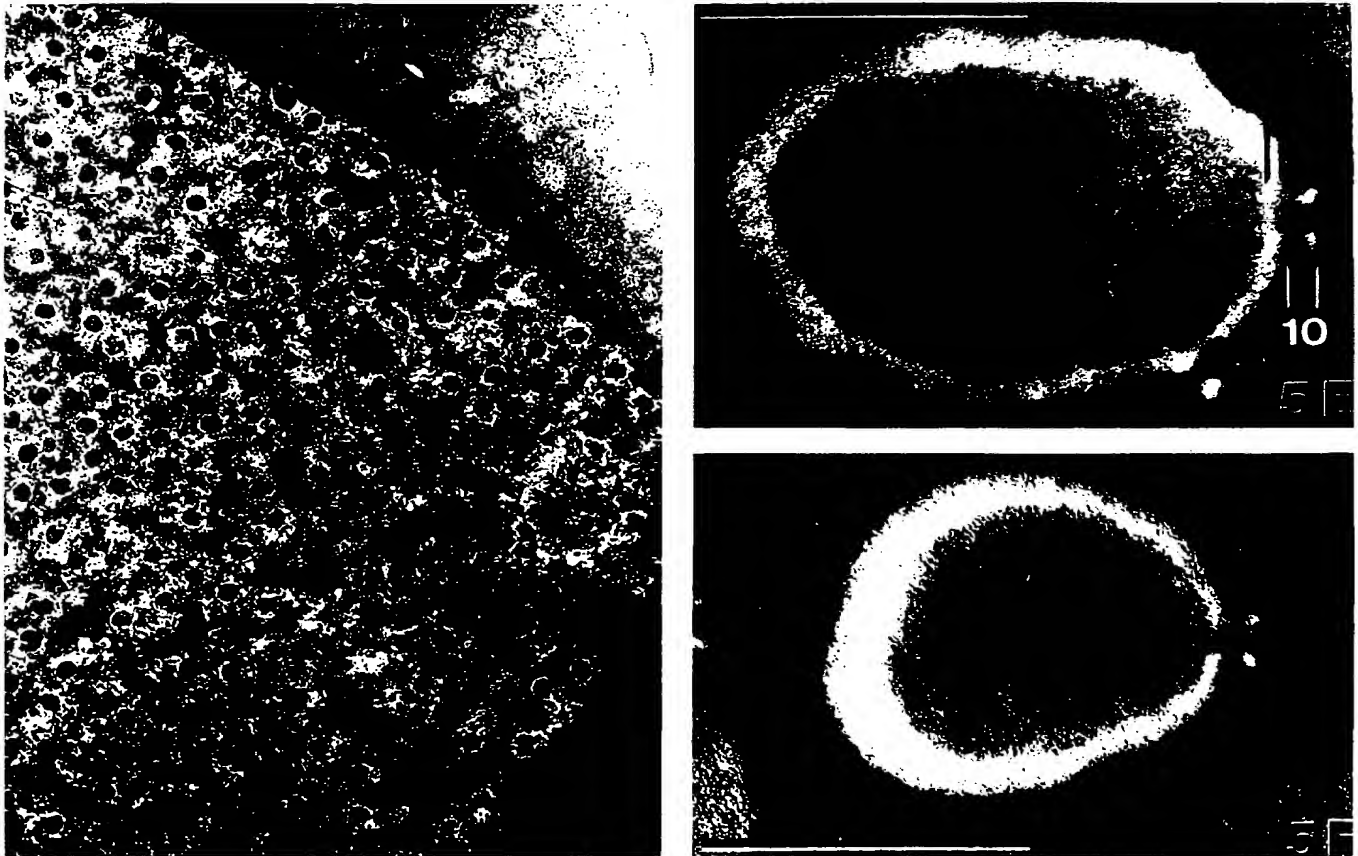


FIG. 4. Electron micrographs of the C5b-9 or membrane attack complex lesions. **Left:** A negatively stained erythrocyte membrane after complement attack, demonstrating innumerable discrete "holes." (Courtesy of Dr. R. Dourmashkin.) **Right:** A single complex penetrating a liposome. The long axis of the complex is perpendicular to the lipid surface and the hydrophobic domain is inserted 4 to 5 nm through the lipid milieu. The complex projects into the aqueous phase approximately 10 nm and displays a thickened outer annulus. (From Bhakdi and Tranum-Jensen, ref. 99, with permission.)

the hydrophobic milieu. C5b678 complexes in membranes bind C9 with extremely high affinity and with stoichiometries consistent with poly C9 formation (12–15C9:1C8). The resultant complexes appear to contain C5b and the C8 β chain in a rodlike 17-nm projection, while C6, C7, C8 α - γ , and C9 are associated with the tubular lesion itself.

The formation of tubular poly C9 is not, however, a *sine qua non* for expression of the lytic properties of C5b-9 complexes. Functional transmembrane lesions are formed by complexes bearing numbers of C9 molecules well below that required for poly C9 formation, and the functional diameter of such channels is roughly proportional to the number of C9 molecules in the complexes (100). Furthermore, thrombin-cleaved C9, which cannot form poly C9, remains capable of supporting complement-mediated cytolysis. Thus while poly C9 formation may have an important role in the genesis of the classical MAC structure, the nature of the minimal effective lytic C5b-9 complex remains to be resolved.

CELLULAR COMPLEMENT RECEPTORS

Many of the biologic functions of complement are mediated by interaction of complement cleavage fragments with specific cell membrane receptors. The interaction of these complement component products with their receptors triggers a series of complex biochemical responses within the cells (101–103). Some of these fragments, such as C3b, iC3b, and C3dg, are bound to the site of complement activation on a target particle. The interactions of these target-bound components with their cellular receptors is thought to trigger a number of specific responses such as phagocytosis by neutrophils and macrophages, or B lymphocyte activation. Complement activation also generates several smaller peptides, C3a, C4a, and C5a, that are released into serum or extracellular fluid. This group of three polypeptides constitutes the complement-derived anaphylatoxins.

Anaphylatoxins and Their Receptors

The binding of these released polypeptides to specific cell surface receptors leads to a series of cellular responses important to the initiation and maintenance of the inflammatory process.

C3a is released on cleavage of C3 by either the classical or the alternative pathway C3 convertase. It is a 9,000-dalton, nonglycosylated protein with a *pI* of 9.7. C3a constitutes the N terminal 77 amino acids of the C3 α chain (104,105). Receptors for C3a have been identified on mast cells and basophils, smooth muscle cells, lymphocytes, and perhaps platelets. Engagement of the C3a receptors on mast cells or basophils induces degranulation, with release of histamine and other mediators of anaphylaxis; thus the name anaphylatoxin. Binding of C3a to tissue preparations induces contraction of smooth muscle cells. Whether smooth muscle contraction is mediated directly, or secondarily by histamine, is unclear. Although ileal

contraction in response to C3a can be blocked by antihistamines, uterine contraction cannot (105,106). *In vitro* studies have also shown that C3a induces the secretion of mucus by goblet cells, another important feature of allergic and anaphylatoxic responses.

C5a, the "classic" complement anaphylatoxin, is an 11,000-dalton protein that represents the N terminal 74 amino acids of the C5 α chain. Approximately 25% of its MW is contributed by a single asparagine-linked oligosaccharide (104,105). Interestingly, the human peptide is heavily glycosylated but C5a from certain other species, such as the pig, has little or no carbohydrate. The function of the carbohydrate is unknown. In many systems removal of the carbohydrate leaves the activity of C5a unaltered. C5a is approximately 200-fold more potent as an anaphylatoxin than is C3a. Part of its anaphylatoxic effect is mediated by direct binding to a specific receptor on basophils and presumably mast cells. Part of its effect is indirect, mediated via binding to neutrophils which presumably then release a mast cell degranulation-inducing substance. C5a and C3a both show tachyphylaxis. Cells stimulated with one of these peptides will respond less well to a second stimulation with the same molecule. They will, however, respond to stimulation by the other anaphylatoxin. This is taken to indicate that each peptide binds to its own distinct and specific receptor. In addition to its anaphylatoxic effects on mast cells and smooth muscle, C5a also has histamine-independent effects on endothelium, causing increased vascular permeability. C5a also induces the directed locomotion (chemotaxis) of neutrophils and monocytes. The chemotactic receptor on these cells for C5a has been well studied (107). Binding of C5a to its receptor has a dissociation constant of 2 to 3 nM, and there are about 2×10^5 C5a receptors per neutrophil (108). In its ability to promote chemotaxis, C5a is quite different from C3a, which apparently has no effect on leukocyte chemotaxis. C5a has a number of other important effects upon neutrophils, causing increased adhesiveness, aggregation, and adherence to endothelium, and also triggering both degranulation and oxidative burst activity (109).

C3a and C5a have also recently been shown to have important and opposite effects on *in vitro* immunoglobulin production by human lymphocytes. C3a suppresses and C5a enhances immunoglobulin secretion; both apparently act at the level of the T cell, presumably through receptor-mediated mechanisms.

A third peptide generated during complement activation, C4a, has only recently been shown to possess anaphylatoxic activity. It is a nonglycosylated, cationic peptide of 8,650 daltons. It is 100-fold less potent as an anaphylatoxin than C3a and 25,000-fold less potent than C5a. Its potential effects on neutrophils and lymphocytes have not been examined. Interestingly, C4a can elicit cross-tachyphylaxis to C3a, but not C5a, in guinea pig ileum. This suggests that C4a and C3a may act through the same receptor.

All the complement anaphylatoxins are rapidly cleaved in serum by the action of carboxypeptidase N, which removes the carboxy terminal arginine shared by all three molecules (104,105). The loss of the C terminal arginine

destroys the anaphylatoxic activity of all three molecules. However, C5a_{des arg}, although about 10-fold less potent than native C5a, does retain some chemotactic activity. A 60,000-dalton noncomplement serum protein called helper factor has been identified which, on binding to C5a_{des arg}, further increases its chemotactic activity without restoring anaphylatoxin activity (109). This protein may act to distort or obscure the carbohydrate present on C5a_{des arg} since deglycosylated C5a_{des arg} has been shown to be 10-fold better as a chemoattractant than unmodified C5a_{des arg}.

Integral Membrane Proteins that Regulate Complement Activity

One of the most important developments in complement research over the past 20 years has been the recognition of a series of integral membrane proteins present on cellular surfaces that regulate complement activation, degradation, and biologic activity. In many cases these proteins also function as membrane receptors. The binding of ligand to these receptors influences the activity or state of differentiation of the cells on which they reside.

The Receptors for Target-Bound Fragments of C3: CR1, CR2, CR3, and CR4

The best-studied cellular receptors for complement components are those involved in complement enhancement of the phagocytosis of bacteria, yeast, antibody-sensitized erythrocytes, and other particles. It has been shown that receptors for several C3 fragments are of great importance in phagocytosis. The discovery of cellular complement receptors is credited to Nelson in 1950. Although earlier reports are replete with examples of fresh serum promoting binding of particles to phagocytic cells, Nelson showed that neutrophil phagocytosis of *Treponema pallidum* and *Streptococcus pneumoniae* sensitized by antibody and complement was more efficient in the presence of human erythrocytes (110). This erythrocyte enhancement of phagocytosis was thought to occur because complement-coated bacteria were immobilized on the surface of the red cells and thus more easily engulfed. Ultimately, this red cell binding of bacteria was shown to occur because of the interaction of organism-bound C3b with a specific C3b receptor on the erythrocytes, a phenomenon known as immune adherence. All primate erythrocytes possess immune adherence (C3b) receptors; although nonprimate erythrocytes do not have this receptor, a functionally equivalent receptor exists on the platelets of nonprimate vertebrate species. Receptors for C3b have also been demonstrated to exist on B lymphocytes, some T lymphocytes, monocytes, and neutrophils as well as mast cells, eosinophils, basophils, and glomerular podocytes (102). The C3b receptors on all blood cells are antigenically identical but the receptor isolated from granulocytes has a slightly higher molecular weight on gel electrophoresis. The isolated C3b receptor glyco-

protein is found in a series of genetically determined polymorphic forms and varies in size from 190 to 280 kd (see section on molecular biology). Its configuration in the cell membrane is unknown, although some evidence suggests that it exists in the form of a hexamer or pentamer. The C3b receptor, in order to distinguish it from membrane receptors for other C3 fragments, is termed CR1. There are 300 to 1,000 CR1 on each erythrocyte and about 5,000 to 30,000 CR1 per cell for the various leukocytes, as determined by binding of specific anti-receptor monoclonal antibody. Since there are about 1,000 times as many erythrocytes as leukocytes in the blood, this implies that over 90% of circulating CR1 are on erythrocytes. CR1 binds C3b with a much higher affinity than native C3, perhaps as much as 1,000-fold higher (63). Hence, the interaction of a C3b-coated particle or immune complex with the C3b receptor is not blocked by free serum C3, allowing C3b to play an important role in phagocytic processes and the catabolism of immune complexes. CR1 is freely diffusible in the membrane of all unactivated cells and when cells are extracted with nonionic detergents CR1 remains in the detergent-soluble fraction, suggesting that it is not associated with cytoskeletal elements. Presumably, in erythrocytes the molecule retains this configuration. On phagocytes, activation of the cell with lymphokines, phorbol esters, or a number of other soluble immunomodulators leads to clustering and ultimately capping of the receptor. It becomes detergent-insoluble, suggesting cytoskeletal attachment (111). Moreover, the receptor is endocytosed. Some data suggest that such endocytosis actually represents cycling of the receptor to an internal pool with subsequent return of the receptor to cell surface (112). If monomer ligand (C3b) is bound to the receptor, it too returns to the cell surface in unaltered form. However, internalized dimeric or multimeric C3b is transferred from the CR1-containing compartment to other compartments within the cell, where it is ultimately catabolized. Evidence obtained with phorbol ester-stimulated cells suggests that CR1 is rapidly phosphorylated and that phosphorylation is reversed after 30 min of incubation with the stimulant. Other stimuli, like the chemotactic peptide f-met-leu-phe, also cause phosphorylation although, unlike phorbol esters, they do not induce ingestion of targets such as C3b-coated red cells.

The binding of a C3b-coated target to CR1 does not itself initiate phagocytosis by a resting phagocyte. A second signal is generally required to initiate the phagocytic process (113). This signal can be provided by a few molecules of IgG bound to the target interacting with IgG Fc receptors on the cell membrane. It can also be provided by various activation signals that appear to take the cell from a resting state to an activated state. Such activation signals can be provided by factors released during chronic infection, fibronectin or laminin acting on mononuclear phagocytes, activators of the phosphoinositide metabolic pathway, and phorbol esters (103).

In addition to its role in phagocytosis, CR1 plays a critical role in the C3 degradative pathway. Upon binding of C3b to CR1, it becomes accessible to the action of factor I. In this respect it acts like fluid-phase factor H; however, the product of this interaction is not the fragment iC3b

mentioned earlier (see Fig. 3). In contrast, cleavage proceeds beyond the iC3b step to yield a further degradative product, C3dg (102,114). CR1 also facilitates the degradation of C4b by factor I, leading to the formation of a cleavage fragment, C4d, bound to the target surfaces. CR1 is believed to play a critical role in the processing of immune complexes. These appear to arise in the circulation quite commonly, for example, during the course of a viral infection, and are potentially quite toxic. It appears that the binding of C3b to these immune complexes is important in their ultimate removal from the circulation. Upon activation, C3b tends to bind to immunoglobulin (see previous discussion). Once immunoglobulin bound, the C3b tends to resist further degradation by factors H and I (71,114). The C3b will interact with adjacent cells with C3b receptors, and the bulk of CR1 receptors in the circulation are on erythrocytes. Such interaction leads to effective removal of the immune complexes from the plasma by adsorption to the erythrocyte surface, and the complexes can no longer diffuse from the intravascular compartment into tissue sites to induce damage. The C3b-containing immune complexes bound to erythrocytes circulate to the liver where they are stripped off the erythrocytes by macrophages within the hepatic sinusoid (115). The erythrocytes, now free of immune complexes, return to the circulation where they continue to circulate with a normal half-life. Interestingly, they appear to lose some membrane CR1 in this process, and states associated with the presence of high levels of circulating immune complexes are characterized by circulating erythrocytes with decreased numbers of CR1 (116).

CR3, CR4, and CD18 Family of Proteins

The product of C3b cleavage by factors H and I is a three-chain molecule, iC3b (see Fig. 3). There are receptors for iC3b on a variety of cell types including PMNs, monocytes, lymphocytes responsible for antibody-dependent and NK cellular cytotoxic activity, and mast cells (102,103). The iC3b receptor (termed CR3 or Mac-1) is functionally the best characterized of all the complement receptors. It is a two-chain molecule with a 150-kd α chain and a 95-kd β chain. This receptor is part of a family of such proteins, each with a distinct α chain, which all share the same β chain. Other members of this family (CD18) include LFA-1 (lymphocyte function antigen-1) and p 150-95, another C3-binding molecule now thought to represent CR4. The function of the β chain appears to be to direct insertion of the complex into the cell membrane (117). A portion of the cellular CR3 content resides within granules in neutrophils and is translocated to the cell surface when the cell is activated. The location of the receptor within the neutrophil has been reported by many workers to be the specific granule. However, a portion of the receptors may reside in another, more poorly defined, granule compartment that also contains the enzyme gelatinase. CR3 is quite specific in terms of ligand binding; in the presence of Ca^{2+} and Mg^{2+} ions, it will bind iC3b but does not recognize C3b. C3dg or C3d are bound weakly. It is re-

ported that the receptor recognizes in part a characteristic arginine, glycine, asparagine (RGD) sequence in the α chain of C3, but that yet another binding site on the molecule is required for stable binding. The receptor is also reported to have conglutinin-like properties, binding to carbohydrate on C3 via a lectinlike interaction. This may represent the additional binding site (103). Like many receptors that recognize the RGD sequence, this receptor is reported to be important in cellular adherence and cells deficient in the receptor have a major adherence defect. A group of children have been reported who lack or have very low levels of the CD18 family of glycoproteins on their cell membranes. The phagocytes of these children ingest particulate targets poorly and also have multiple defects in other adherence-dependent functions. As a result of this deficiency, the children have frequent soft tissue and cutaneous infections with a variety of bacterial pathogens, especially staphylococci and *Pseudomonas aeruginosa* (118). Ligands that interact with proteins of the CD18 family are reported to appear on endothelial cells when these cells are treated with certain lymphokines, suggesting that the release of lymphokines facilitates the attachment of immune effector cells to endothelium, which is the first step in emigration into areas of tissue inflammation. In the phagocytic process itself, CR3 functions much like CR1, requiring a second signal for phagocytosis in the resting cell.

CR2 and Other Complement Peptide Receptors

This 140-kd integral membrane protein recognizes the physiologic C3 product formed upon interaction of CR1 with C3b and factor I, C3dg. Elastase and other tryptic enzymes can cleave C3dg to a further degradative fragment, C3d, which is also recognized by CR2. This integral membrane protein is present on all B lymphocytes and epithelial cells and is reported to be important in providing the B lymphocyte with signals that stimulate cell cycling and differentiation (119). CR2 also serves as the cellular target for the binding of Epstein-Barr virus to B lymphocytes. Its presence on epithelial cells is believed to be important in initial Epstein-Barr virus infections in which epithelial cells of the pharynx are first invaded. Although CR2 is not found on phagocytes, the C3dg or C3d fragments on a particulate target can facilitate binding and ingestion via interactions with CR3 and CR4.

There are receptors for several other complement components that have been identified on various cell types, but their biologic importance is not yet understood. A receptor for factor H has been reported on B lymphocytes and perhaps granulocytes and monocytes. Preliminary characterization of this receptor has shown it to migrate as a single 50,000-dalton band on SDS-PAGE analysis under reducing conditions (120). Binding of factor H to lymphocytes via this receptor is said to trigger factor I release from the cells, and exposure of monocytes to factor H increased NBT reduction and chemiluminescence. Lymphocytes, PMNs, and platelets also possess a receptor for C1q (121). There may be as many as 10^6 C1q re-

ceptors per neutrophil. The physiologic significance of this receptor is also unknown, but it is tempting to speculate that it may have a role in adherence of classical pathway activators to phagocytic cells. Since the C1 inhibitor binds to C1r and C1s and causes their release from macromolecular C1, C1q may remain attached to the classical pathway activator and be exposed to cellular receptors. Interaction of C1q with its receptor on monocytes and macrophages has also been shown to activate the cell to facilitate phagocytosis of both IgG-coated ligands and C3b/C4b-coated ligands. A macrophage receptor for Bb, which leads to macrophage spreading on glass or plastic surfaces, has also been reported.

Decay-Activating Factor (DAF), Homologous Restriction Factor (HRF), and Membrane Cofactor Protein (MCP)

These three membrane proteins do not act as typical receptors in that they do not promote the binding of a complement-coated target to the surface of cells expressing these molecules (although evidence is accruing that, in certain cases, cellular activation signals may be delivered by ligation of these glycoproteins). Nevertheless, these molecules do interact specifically with complement activation products and they play an essential role in preserving cellular integrity, preventing lysis of innocent bystander host cells resulting from spontaneous or induced complement activation.

Decay-accelerating factor (DAF) is a 70-kd membrane protein that is linked to the cell membrane by the diacylglycerol moiety of a phosphatidylinositol molecule which is covalently attached via a glycosidic linkage to the carboxy terminus of the protein (122). DAF inhibits formation of the classical pathway C4b2a convertase by interacting with C4b and preventing C2 binding, and also by destabilizing the convertase once it forms and enhancing its rate of decay. It also inhibits formation and promotes decay of the alternative pathway convertase, although it is somewhat less effective in this regard. DAF does not act as cofactor for cleavage of C3b or C4b (123). The molecule is present in the membrane of all blood cells and endothelial cells and exists in two forms, which differ in size and which are thought to result from alternative splicing of the terminal portion of the gene encoding DAF (124). One form of the protein is processed to provide the phosphoinositide membrane linkage and the other is truncated and released from the cell without the membrane anchor moiety. It is also reported that the form of this protein found in neutrophils is slightly heavier than that found in erythrocytes. Like HRF, this phospholipid-anchored membrane-protective molecule is missing from the cells in patients with the disease paroxysmal nocturnal hemoglobinuria (PNH) (125,126). These abnormalities appear to account for at least a portion of the exquisite complement sensitivity of erythrocytes in PNH and are likely responsible for chronic intravascular hemolysis in this disease.

Homologous restriction factor is a 65-kd regulatory pro-

tein which is also phospholipid anchored and has been studied thus far on lymphocytes and erythrocytes, although it has been shown to be present on monocytes, neutrophils, and platelets as well (127,128). It interacts with both C8 and C9 of the membrane attack complex to prevent successful insertion of the complex through the membrane bilayer, thus protecting cells from the late-acting steps in complement attack. It derives its unusual name from the observation that, in the case of each species thus far studied, the factor recognizes and interacts with homologous C8 and C9 far better than heterologous C8 and C9. The presence of this factor in cell membranes would appear to explain the fact that complement proteins are far more effective at lysing cells of heterologous species than they are at lysing cells of the homologous species. HRF is also reported to inhibit the action of the cellular cytotoxin termed perforin or cytolyisin, a molecule found in large granular lymphocytes which has significant homology to C9 and which has been reported to cause cell lysis (129).

Yet another membrane protein termed membrane cofactor protein (MCP) or gp45-70 has been reported in the membranes of most blood cell types, but not erythrocytes. In addition, it is reported to be present on epithelial cells, fibroblasts, and endothelial cells. This protein binds to C3b and iC3b but does not appear to have sufficient affinity to act as a complement receptor per se. It does, however, facilitate the factor I-dependent degradation of C3b to iC3b and may in fact be more efficient in this regard than any of the previously identified fluid-phase or membrane cofactors (130). Interestingly, it does not have decay-accelerating activity for either the C3 or C5 convertases.

MOLECULAR BIOLOGY, SYNTHESIS, AND DEFICIENCIES OF COMPLEMENT PROTEINS

Within the past decade the molecular biology of the complement system has come under intense scrutiny. DNA encoding nearly every component of the cascade, as well as regulatory factors and cellular receptors, has been cloned and sequenced. Chromosomal mapping of the complement genes and studies of the regulation of synthesis of several components have been performed with molecular probes. An encyclopedic review of these data is beyond the scope of this chapter and is available elsewhere (131). We treat several major themes which have emerged from these studies.

The C3b/C4b Binding Protein Superfamily

A large number of plasma and membrane proteins interact with C3b or C4b. These proteins are important in complement activation, regulation of the cascade, or as cellular receptors. They include factor H, C4BP, CR1, CR2, DAF, gp45-70 (MCP), C2, and factor B (132,133). A close relationship between these proteins was first suggested for the regulatory plasma proteins H and C4BP,

and the regulatory and membrane receptor protein CR1, on the basis of classical genetic techniques. By studies of electrophoretic polymorphic variants, the genes for H, CR1, and C4BP were found to be very closely linked in the human. As molecular probes and eventually full-length cDNA clones for these proteins have become available, a gene superfamily has emerged. At least the CR1, CR2, C4BP, H, and DAF genes are located on the long arm of chromosome one in the human (134). More importantly, each member of this group has been shown to contain from 8 to over 30 short homologous repeating units approximately 60 amino acids in length. These repeating units generally appear in sequence and occupy the amino terminal portion of the protein (133). There are eight or nine highly characteristic conserved amino acids, including four cysteines, as well as conserved hydrophobic regions. The deduced structure of CR1 has shown its 28 to 33 short consensus repeats to be organized into longer homologous repeats, each of which contains seven of the shorter segments. Duplication en bloc of one of these longer homologous repeating segments is thought to have given rise to at least one of the more common electrophoretic variants (103,131). The deduced structure of CR2 appears similar. The CR1, CR2, C4BP, and DAF genes are very closely spaced and share a higher degree of homology, while factor H is not as closely linked and is also more dissimilar in primary sequence (134). The C3b/C4b binding capacity and cofactor activity of these proteins have been explicitly shown to reside in the repeating units in the case of H and C4BP, and this relationship is presumed to hold true for the remainder of the family. While less well studied, the organization and structure of the murine C3b/C4b binding protein genes appear similar.

C2 and factor B, which also bind and interact with C4b or C3b, each contain three of the typical short consensus repeats at their amino termini (133,135). The carboxy terminal ends of these proteins confer serine protease activity. C2 and factor B are encoded on chromosome 6 in the human, within the major histocompatibility complex (MHC), and are discussed further later. Two other complement components, C1r and C1s, have also been found to have two of the homologous repeating units at the carboxy terminal end of their noncatalytic heavy chains. Several noncomplement proteins: β_2 -glycoprotein 1, factor XIII, and the (p55 chain of) IL-2 receptor also contain

several copies of the short homologous repeating unit. Careful studies of genomic clones for several proteins in this superfamily indicate that each short repeat may be encoded by a discrete exon. The functional significance of these structures and the factors underlying the presumptive multiple gene duplications responsible for this family remain to be determined.

The MHC-Linked Complement Genes

The genes encoding C2, factor B, and C4 lie in a 120-kb stretch of DNA between the HLA-DR and HLA-B loci on chromosome 6 in the human (131) (Fig. 5). The C2 and factor B genes are less than 1 kb apart. These two genes demonstrate high primary sequence homology and each encodes several amino terminal short homologous repeats linked to a carboxy terminal serine protease domain as described previously. The exon structure of the DNA encoding the protease domain of factor B and C2 is similar, but not identical, to that of other serine proteases. The C2 and factor B genes contain a unique exon encoding a region of polypeptide chain between the Asp and Ser residues of the active site that is not found in other serine proteases and share an overall 33% homology in their enzymatic domains. Thus C2 and factor B almost certainly represent a gene duplication (135).

Approximately 30 kb distant from the C2 and factor B genes lie two separate C4 loci, each associated with a gene encoding the noncomplement protein steroid 21-hydroxylase. The two C4 genes encode the two isotypic variants C4A and C4B. Fragments of C4A and C4B deposited on red cells are responsible for the HLA-linked Rodgers and Chido blood group antigens, respectively. The homology between C4A and C4B is approximately 99%, with perhaps as few as six amino acid substitutions defining the two isotypes. These isotypic variations are clustered in the portion of the C4 molecule which gives rise to the C4d fragment and contains the reactive thioester site (136). The functional consequence of the substitutions appears to be a predilection of C4A to form amide bonds with proteins, whereas C4B preferentially forms ester bonds with carbohydrate moieties. In studies of erythrocyte lysis, C4B is far more active than C4A. Multiple allotypic variants of each C4 isotype exist, so that a normal com-

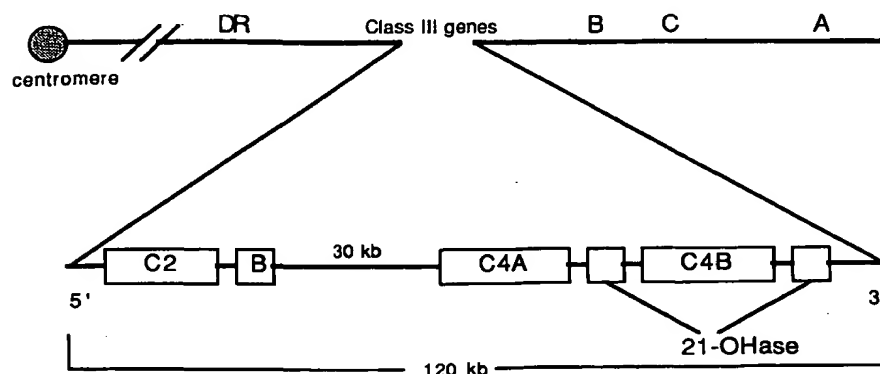


FIG. 5. A map of the MHC-linked complement genes (class III MHC gene products) on the short arm of chromosome 6 in the human. The arrangement of the HLA, A, B, C, and DR loci is shown relative to the complement genes. The expanded 120-kb segment shows the relative position and size of the C2, factor B, C4A and B, and 21-hydroxylase (21-OHase) genes. Not drawn strictly to scale.

plement of C4 genes may encode up to four distinct forms of the protein. Furthermore, null alleles at both loci are fairly common, and 10 to 15% of the normal population may carry at least one null (or Q0) C4 allele. About half of null alleles thus far examined result from large deletions which involve most or all of one C4 gene and an adjacent 21-hydroxylase gene. While less well documented, evidence is also accruing that a significant number of haplotypes in the general population may contain three C4 loci. The high frequency of both large deletions and apparent duplications has led to the proposal that unequal crossover events between the C4 loci of sister chromatids during meiosis may be responsible for such haplotypes with one or three C4 genes (137). In the mouse, only one functional C4 product is found. A second C4-like protein is encoded in the major histocompatibility complex, and expression of this protein is under the control of sex hormones (sex-limited protein or SLP). Absence of C4 function in SLP is probably related to accumulated sequence changes near the C1s cleavage site (131).

Because of their proximity in the genome, specific combinations of C2, factor B, and C4 allelic variants tend to occur together. Crossovers within these combinations are uncommon, and they tend to be inherited as units referred to as complotypes (131). Complotypes can serve as markers for even larger units which are called "extended haplotypes" and include the HLA A, B, C, and DR loci. These are usually inherited en bloc and occur in the general population at frequencies higher than those predicted by their physical proximity alone. It has been suggested that crossovers within extended haplotypes are actively suppressed, but the mechanisms and selective pressures responsible for this remain speculative.

Biosynthesis of Complement Components

Over the last several decades there has been a steady accumulation of data relating to the biosynthesis of complement components. In the human, the liver appears to play a preeminent role in the synthesis of most of the components. This has been shown clearly for C3, C6, C8, and factor B by documenting a switch in the circulating allotypes of these proteins to those characteristic of the donor after orthotopic liver transplant (138). Notably, the recipient's complement component allotypes do not disappear completely after such surgery, suggesting extrahepatic synthesis as well. Data regarding the site of synthesis of most other components are derived primarily from *in vitro* studies of primary tissue cultures or established cell lines, both animal and human. On the basis of such information, it appears that functional C1 macromolecules are produced by a variety of epithelial cells of endodermal origin, and these cells, as well as macrophages, are thought to be an important locus of *in vivo* production (139). The source of virtually all the other components studied appear to be the liver or the mononuclear phagocyte system or both (140). While mononuclear phagocytes probably contribute little to total plasma complement levels, their output may be of considerable importance at local sites of inflammation.

Regulation of complement protein biosynthesis is complex. The majority of studies have been carried out with primary macrophage cultures and hepatocyte or macrophage-like cell lines. Synthesis is responsive to a wide variety of modulators including immune complexes, ambient complement protein levels, cytokines, neuropeptides, histamine, and arachidonate metabolites, and the responses evoked are both protein and tissue specific (140). Regulatory mechanisms have been demonstrated at the pretranslational, translational, and post-translational levels in various *in vitro* systems. At the clinical level, the majority of complement proteins are elevated in plasma during acute-phase responses. Careful studies of regulation of C2 and factor B synthesis using molecular probes are in progress. Despite divergent, independent responses of the synthesis of these two proteins to a variety of stimuli, it appears that at least one segment of DNA 3' to the C2 coding sequence is essential for expression of both genes (141). It is interesting to note that at least two complement proteins, C1 and C8, are composed of two or more gene products which are assembled non-covalently in the extracellular compartment after secretion. The plasma pool of these components exists as an equilibrium mixture of complexed and disassembled subcomponents, and synthesis of the various subcomponents is clearly separable in both *in vitro* systems and genetically deficient individuals.

Complement Deficiency States

Complement deficiency states are distinctly uncommon, and this low frequency points to the strong selective pressure favoring maintenance of an effective complement cascade. Complement protein alleles, including null alleles, show codominant behavior. Thus heterozygotes for null alleles have roughly half-normal levels of the components in question and are usually clinically normal (with one exception—see later discussion). The consequences of homozygous deficiency states divide roughly in accordance with the portion of the cascade involved. Humans with deficiencies of the alternative pathway are highly susceptible to a variety of pyogenic bacterial infections (presumably due to failure of C3-dependent opsonization), whereas those with terminal component defects have a poorly understood isolated propensity to disseminated neisserial infections. A substantial proportion of individuals with terminal component deficiencies, especially C9, are, however, entirely well. Autoimmune disease, presenting as glomerulonephritis or systemic lupus erythematosus-like syndromes, is seen in deficiencies affecting all three portions of the complement cascade but is the particular hallmark of classical pathway defects (142,143). Such disorders are seen in over half of individuals with C2 or C4 deficiency. The genesis of this striking relationship is unclear. It has been proposed that C2 and C4 deficiencies profoundly alter the disposition of immune complexes and thereby promote both end-organ pathology and persistent immunoregulatory abnormalities (144). It is notable, however, that heterozygous family

members of C2-deficient patients and humans whose genotype includes even one null allele (especially C4AQ0) among the four C4 genes have increased incidences of autoimmunity (142). This finding, and the relationship of C2 and C4 genes to the MHC loci, has raised the question of additional disease-susceptibility genes linked to the null complement alleles. These questions have not been resolved.

Deficiency of C1 inhibitor in the human is clinically manifest in heterozygotes as the syndrome of hereditary angioedema (145). Affected individuals are subject to recurrent localized soft-tissue swelling. C1 inhibitor is an important regulator of the clotting, kinin-generating, and fibrinolytic enzyme systems in addition to the complement cascade. Chronic consumption of the inhibitor by these enzyme systems outstrips the output directed by one normal gene, and C1-inhibitor titers fall well below half-normal levels. Failure of homeostatic regulation of one or more of the above protease systems is then believed to generate a vasoactive mediator(s) which results in angioedema. The precise mediator has been extensively sought but remains uncertain.

A number of animal models of complement deficiency are available. These include C2-, C3-, and C4-deficient guinea pigs, C3-deficient dogs, C5-deficient mice, and C6-deficient rabbits (146). These have been useful in elucidating the role of complement in opsonization and in confirming abnormalities of the immune response to prototypic antigens. Some of these animals exhibit clinical diseases or subclinical serologic abnormalities that mimic their human counterparts.

CONCLUSION

As recently as 1969, Macfarlane Burnet wrote in his book *Cellular Immunology*:

Since the studies of Bordet, Ehrlich and Wasserman, the concept of complement as an essential part of the mechanism of immunity has progressively been replaced by a rather uncertain decision that the classic phenomenon of complement lysis of red cells is a laboratory artifact of no real significance for immunity.

We hope that it is clear that our understanding of complement has come a long way since that time. We are now certain that the complement proteins play a critical role in host defense and the development of autoimmunity. We believe it likely that these proteins will prove to be important in control of a number of steps in the immune response as well. The fact that very few individuals are missing any of the many proteins and the fact that the proteins show remarkable evolutionary stability suggest that their further study will reveal new important control functions. Now that the chain structure of most of the proteins and cleavage fragments is known and the amino acid sequence of many of the proteins is established, understanding their interactions and the biologic consequences of their activation will provide one of the major challenges of the next decade.

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Immunology

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and generalized swelling. Some of the affected fetuses may be aborted, others may be delivered stillborn, and others still may be born alive but with severe defects, particularly in the central nervous system, where considerable damage is caused by the deposit of bilirubin in ganglia. Haemolytic disease of the newborn does not always develop in every Rh⁻ mother carrying an Rh⁺ fetus. Usually the first pregnancy is not affected, but in later pregnancies, as the titre of Rh antibodies increases, the chances of haemolytic disease of the newborn also increase. The disease can be prevented by giving the mother anti-RhD IgG fraction intramuscularly at the time of delivery (within 60 h). These passively administered antibodies prevent sensitization of the mother's lymphoid system and thus the production of RhD antibodies. The passively administered antibodies are then eliminated by natural decay. This treatment reduces the risk of an anamnestic response during subsequent pregnancies by 95%.

Type III hypersensitivities induced by immune complexes

Principle

The interaction of antigens with their corresponding circulating antibodies leads to the formation of *antigen-antibody (immune) complexes*. Normally, immune complexes are removed from the circulation through the mononuclear phagocyte (reticuloendothelial) system, particularly in the liver (by Kupffer cells), spleen, and lungs, but if they are formed in large quantities, they are deposited in various tissues. The deposited immune complexes bind and activate complement and the C3a and C5a fragments so generated bind to basophils in the blood and cause their degranulation. Immune complexes may also interact directly with basophils and platelets (via the immunoglobulin Fc regions) and cause their degranulation. Some of the released mediators, in particular histamine and 5-hydroxytryptamine, cause retraction of endothelial cells and so increase the permeability of the blood vessels and lead consequently to the deposit of more immune complexes. The activated platelets aggregate and initiate the formation of small clots on the collagen of the exposed basement membrane beneath the endothelial cells. Other mediators attract neutrophils which then attempt to phagocytose the deposited complexes. The tissue-bound complexes cannot be easily engulfed, however, and the macrophages spill their

lysosomal contents over the tissue. Normally, the released lysosomal enzymes would be inactivated quickly by substances in the serum, but since the serum is to a great extent excluded from the contact zone between the phagocytes and the tissue cells, they have enough time to attack the tissue. The resulting tissue damage leads to a form of hypersensitivity which involves IgG rather than IgE antibodies. The hypersensitivity manifests itself in a characteristic tissue response which will be described shortly.

The immune complexes are deposited preferentially in certain sites throughout the body — the kidney glomerulus, the joints, the lungs, and the skin. The reasons for this preference may vary from organ to organ. The deposit of immune complexes in the kidney may occur because the blood pressure in the glomerular capillaries is four times higher than in other capillaries. Also, the glomerulus is a filter through which body fluids have to pass and it may retain immune complexes by a simple filtering effect. For a similar reason, immune complexes may also accumulate on other body filters: the ciliary body of the eye, where aqueous humour forms, and the choroid plexus in the brain, where cerebrospinal fluid is produced. The characteristics of the disease which leads to immune-complex deposits may also determine the site for the deposit. Systemic lupus erythematosus, for example, is characterized by the appearance of DNA-specific antibodies (see Chapter 24) and since DNA has affinity for collagen in the basement membrane of the glomerulus, most of the DNA-anti-DNA complexes accumulate in this organ. Another example is rheumatoid arthritis, in which plasma cells produce Ig-specific antibodies in the synovium of the joint and the immune complexes thus initiate an inflammatory response at this site. Why the deposition of immune complexes only occurs in certain diseases is not known. Possible contributory factors include the affinity of the antibodies and the valency of the antigen (low-affinity antibodies combining with low-valency antigens may form complexes that the body has difficulty clearing); the participation of complement (binding of C3b and C3d to immune complexes solubilizes deposited complexes and the lack of appropriate complement involvement may have the opposite effect); and the nature of the antigen, as was pointed out earlier.

Immune complexes form frequently in autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. The other two situations in which immune complexes may be

involved in the pathogenesis of the disease are, first, low-grade persistent infections such as those characterizing leprosy, malaria, African trypanosomiasis, and viral hepatitis; and second, repeated exposure of body surfaces, such as the lungs, to antigenic material such as pigeon antigens (leading to *pigeon fancier's disease*), or fungi from mouldy hay (leading to *farmer's lung disease*). The experimental models of these two situations are the Arthus reaction and serum sickness.

Arthus reaction

In 1903, N. Maurice Arthus and Maurice Breton described an experiment in which they repeatedly injected normal horse serum subcutaneously into rabbits, with an interval of several days between the individual injections. After the fifth or sixth injection, they observed a skin reaction characterized by firm induration, swelling, abscess formation, and eventual necrosis. It was not necessary that the site of the last injection coincided with that of previous injections; the reaction could be observed at any site where the last injection was made. The phenomenon, now referred to as the *Arthus reaction*, is not peculiar to rabbits; similar reactions were also observed in guinea-pigs, rats, dogs, and humans. The reaction is explained as follows (Fig. 21.17). The repeated injections induce

the formation of precipitating antibodies specific for horse proteins. As the antigen diffuses from the injection site through the tissue and into the regional blood vessels, it combines with the antibodies and insoluble antigen-antibody complexes form locally in the venules. The immune complexes are deposited between and beneath the endothelial cells, where they activate complement. The chemotactic factors liberated from the complement cascade begin to attract neutrophils and platelets to the reaction site. The neutrophils adhere to the tissue-bound immune complexes via their C3 receptors (CR1) and attempt to phagocytose them. Since, however, the complexes are attached to a nonphagocytosable substrate (the basement membrane), the phagocytosis is incomplete and the phagolysosome remains open to the exterior, releasing lysosomal enzymes into the surrounding medium. The released enzymes attack the basement membrane and the tissue surrounding it, collagenases disrupting collagen fibres, neutral proteases destroying the ground substance, and elastases degrading elastic fibres. The proteases also generate C5a from C5, which initiates degranulation of the neutrophils. The mediators released from the granules promote further neutrophil accumulation and degranulation. Some of the mediators act on mast cells and basophils causing their degranulation, thus further exacerbating the inflammatory reaction. Some of the

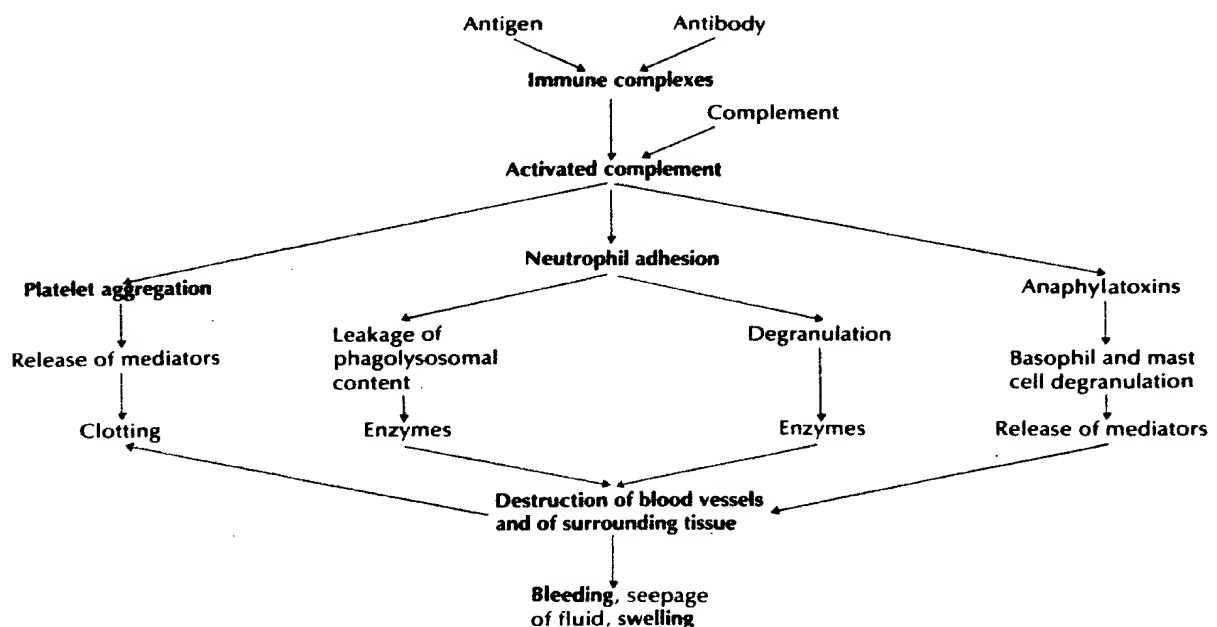


Fig. 21.17 Major mechanisms leading to the Arthus reaction.

Mechanisms of Cell Death Induced by Tumor Necrosis Factor Antagonists

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INTRODUCTION

TNF Antagonists

- Tumor necrosis factor (TNF) antagonists have been shown to be efficacious in the treatment of several autoimmune diseases, including rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, and psoriasis.
- There are currently 2 classes of biologic drugs that target TNF bioavailability: soluble TNF receptors (etanercept) and anti-TNF monoclonal antibodies (adalimumab and infliximab).
- All 3 currently approved agents bear the Fc portion of complement-activating human IgG1; the Fc region is a native component of the monoclonal antibodies, whereas it is genetically fused to the soluble receptor.

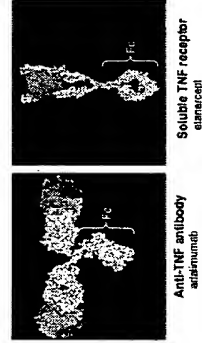
Fc Components of TNF antagonists

- Fc regions bind to Fc receptors (FcR), which are a family of immunoglobulin-binding molecules expressed on immune cells, including macrophages, granulocytes, natural killer cells, B cells, and platelets.
- Antibody-dependent cellular cytotoxicity (ADCC) is mediated by cross-linking of FcR.
- Complement-dependent cytotoxicity (CDC) may be enhanced by the cross-linking of Fc, which increases affinity for the complement component C1q.

OBJECTIVE

To assess the ability of infliximab, adalimumab, and etanercept to bind to membrane-bound (mTNF) and the ability of infliximab and etanercept to induce ADCC and CDC.

Figure 1. Models of TNF Antagonists Bound to TNF



TNF antagonists are shown in white, and TNF trimers are blue, green, and red. The TNF-binding site of the anti-TNF antibody is shown in cyan.

METHODS

- Construction of mTNF-expressing Chinese Hamster Ovary (CHO) cells
 - A mutant TNF that is expressed in membrane-bound form was constructed by site-directed mutagenesis of the wild-type TNF DNA sequence to remove amino acids 77 through 88.
 - The mutated TNF DNA sequence was cloned into a lentiviral expression vector, which was used to transduce CHO cells.
 - CHO cells expressing mTNF were isolated with a fluorescence-activated cell sorter, using a fluorochrome-conjugated anti-TNF antibody.
 - A high-expressor clone (MT-3) was expanded using selective media and used for all experiments.
- Binding of TNF antagonists to mTNF
 - Fluorescein isothiocyanate-conjugated drug (0.3 µg) was incubated with MT-3 cells alone or in the presence of 300-fold excess soluble TNF for 1 hour at 4°C.
 - The cells were washed with flow buffer (phosphate-buffered saline with 0.2% bovine serum albumin) to remove unbound drugs.
 - Binding of TNF antagonists to cells was analyzed using FACSscan.
- ADCC assays (performed in triplicate)
 - MT-3 cells were detached from tissue culture flasks with cell dissociation buffer, washed, and labeled with a membrane integrating dye PKH67.
 - MT-3 cells (0.5×10^6 cells) were incubated with varying concentrations of anti-TNF molecules at 4°C for 30 minutes.
 - Purified donor peripheral blood mononuclear cells were mixed with the treated cells at 10:1 or 40:1 ratios for 4 hours.
 - Propidium iodide (binds to the chromatin of dead or dying cells) was added to each well.
 - The degree of cell death was measured by flow cytometry based on the degree of fluorescence from propidium iodide bound to cellular chromatin.
- CDC assays (performed in triplicate)
 - MT-3 cells were detached from tissue culture plates using cell dissociation buffer to form a single-cell suspension.
 - MT-3 cells (0.5×10^6 cells) were incubated in the presence of etanercept or infliximab at the indicated concentrations for 1 hour at 4°C to allow binding to mTNF.
 - Heat-inactivated fetal bovine serum (negative control), complement component C5-depleted human serum (negative control), or human complement-rich serum was added at a final concentration of 10%.
 - Cells and TNF antagonists were incubated in the presence of complement for 3 hours at 37°C.
 - The degree of cell death was determined by analysis of propidium iodide uptake using flow cytometry.

RESULTS

Figure 2. Binding of Etanercept, Adalimumab, and Infliximab to mTNF

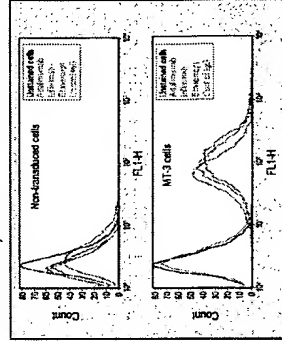


Figure 3. ADCC by Etanercept and Infliximab

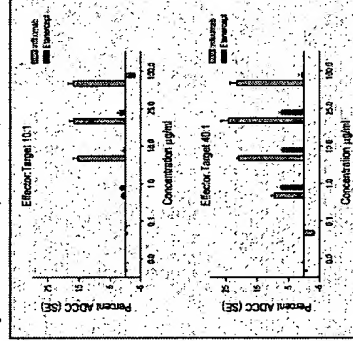
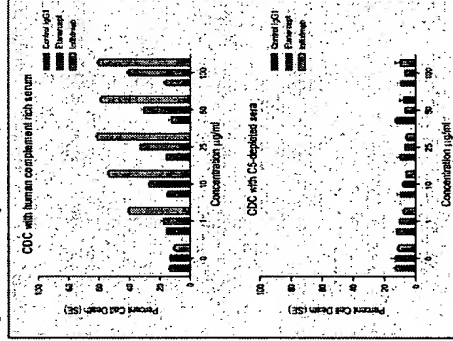


Figure 4. CDC by Etanercept and Infliximab



DISCUSSION

- Soluble receptors and monoclonal antibodies that target TNF decrease levels of bioactive TNF. Both classes of drugs demonstrate efficacy in the treatment of several autoimmune diseases.
- In prior studies, we found that both classes of TNF antagonists bound poorly to FcR and C1q on cells. In the presence of TNF, anti-TNF monoclonal antibodies, but not the soluble TNF receptor, increased binding to FcR and C1q.
- Current experiments showed that the anti-TNF antibody infliximab was more effective at inducing ADCC and CDC pathways in mTNF-expressing cells than the soluble TNF receptor etanercept, perhaps via binding to FcR and C1q.
- These differences in the ability to induce ADCC and CDC pathways may explain the varying spectrum of disease states for which these agents are effective treatments.
- Furthermore, these differences may contribute to the higher rates of fungal and granulomatous infections, such as tuberculosis, observed with infliximab compared with etanercept.^{1,2}

CONCLUSIONS

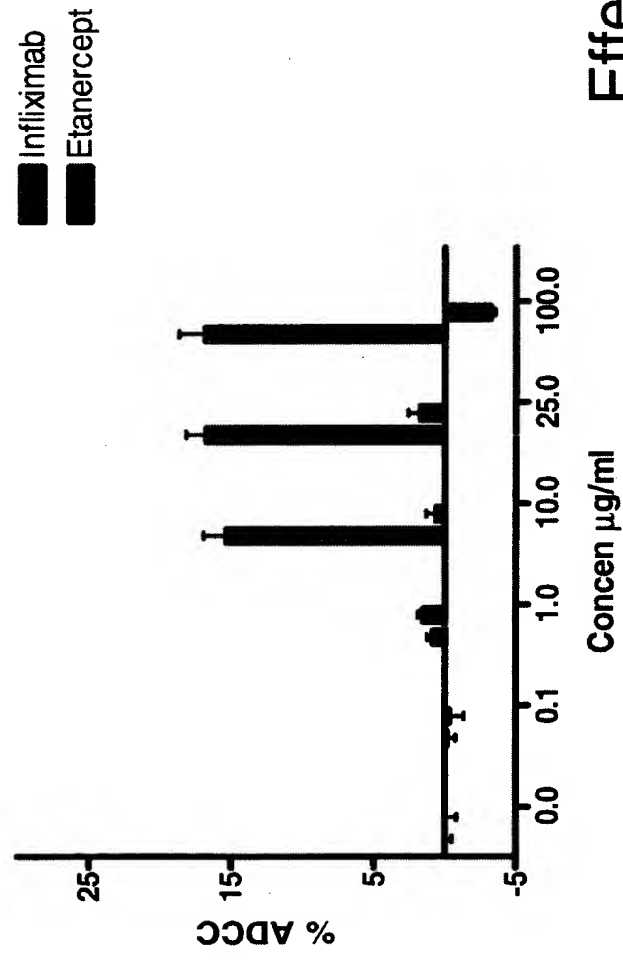
- Both of the TNF antagonists tested, the soluble TNF receptor etanercept as well as the anti-TNF monoclonal antibody infliximab, bound to mTNF on CHO cells.
- Infliximab induced ADCC at lower effector:target ratios than etanercept.
- Infliximab was more effective than etanercept at inducing CDC in mTNF-expressing cells.

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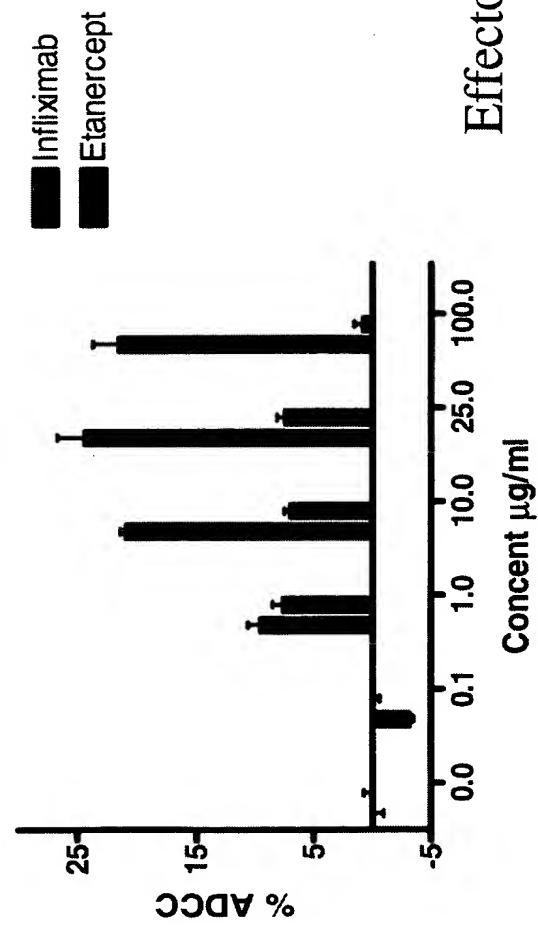
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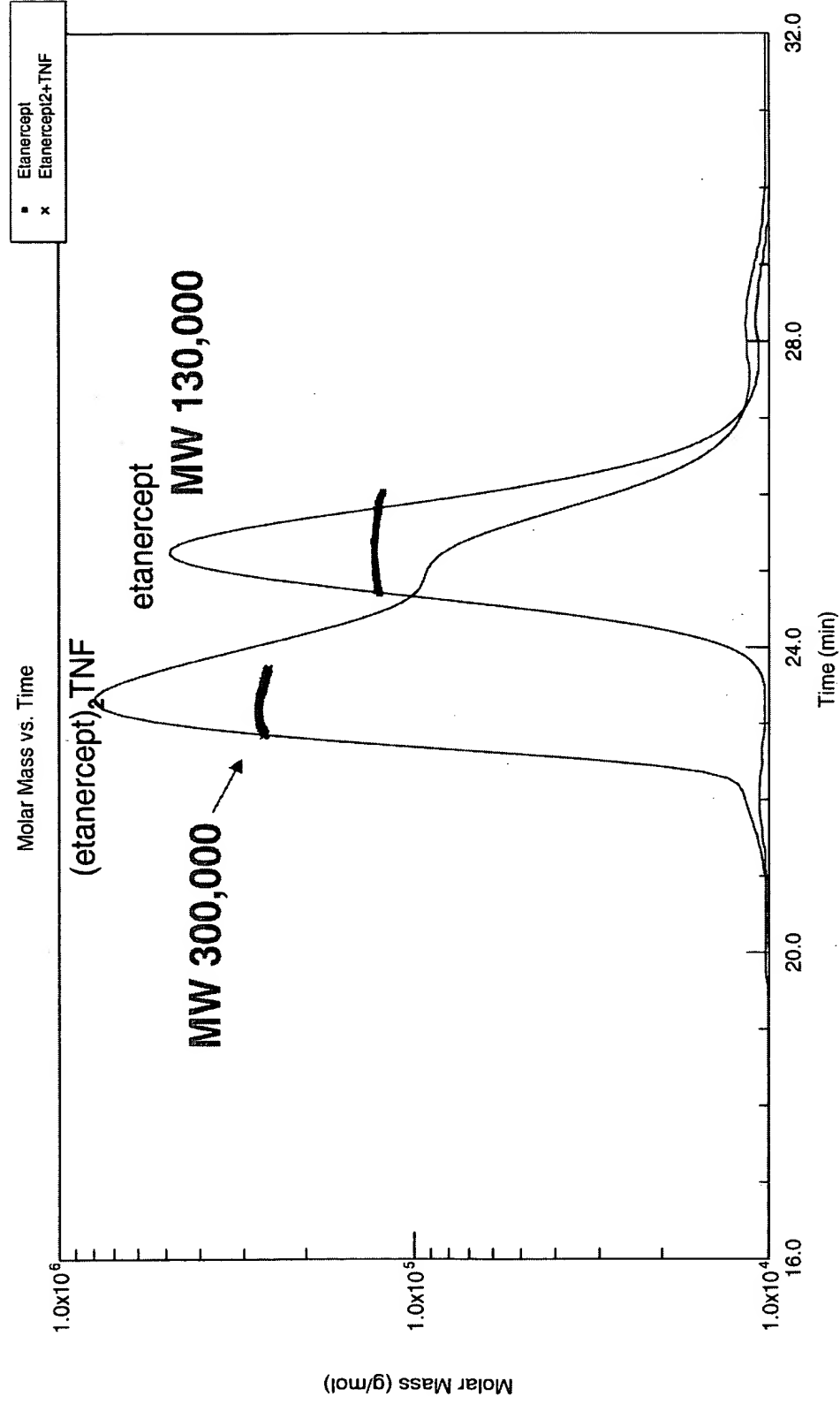
Thanks to Randall Kachem for molecular imaging studies. Research funded by Amgen Corporation, a wholly-owned subsidiary of Amgen Inc., and by Wyeth Research.

ADCC by Etanercept and Infliximab



ADCC by Etanercept and Infliximab

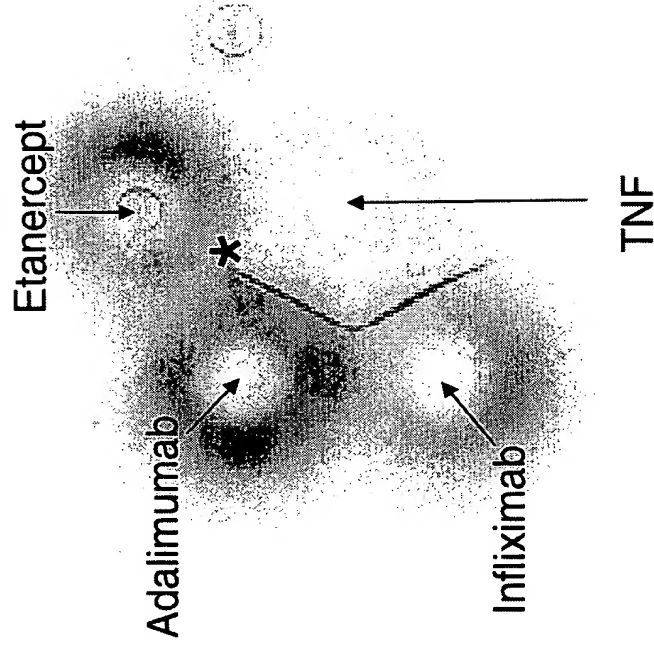
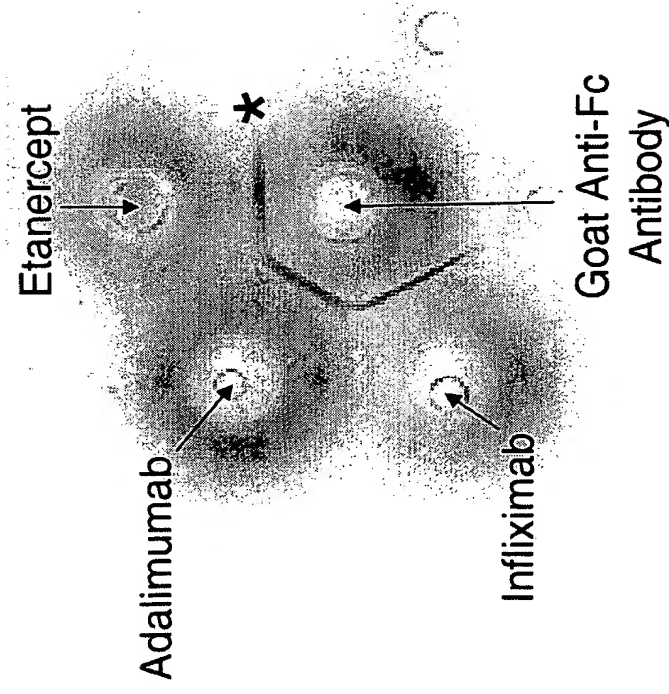




SEC-LS analysis of etanercept-TNF complexes

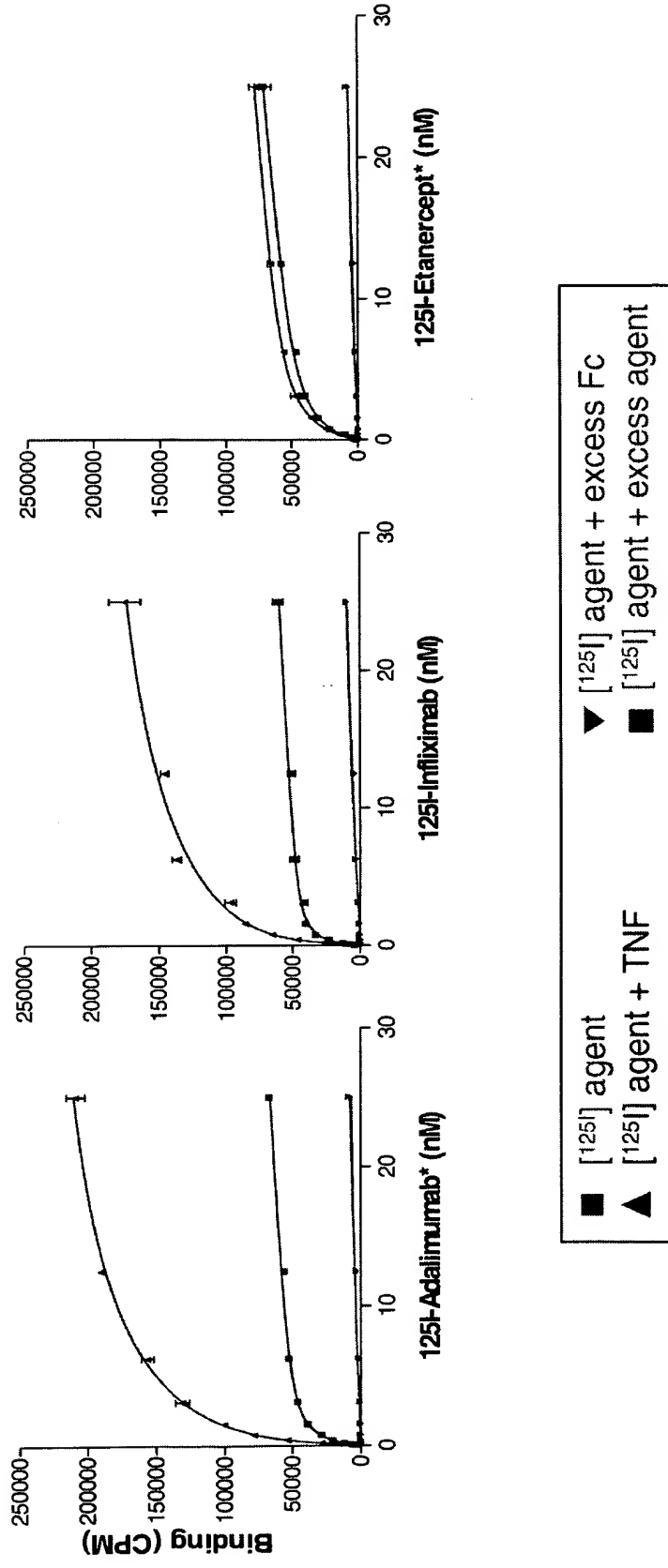
A. Control experiment

B. Test experiment

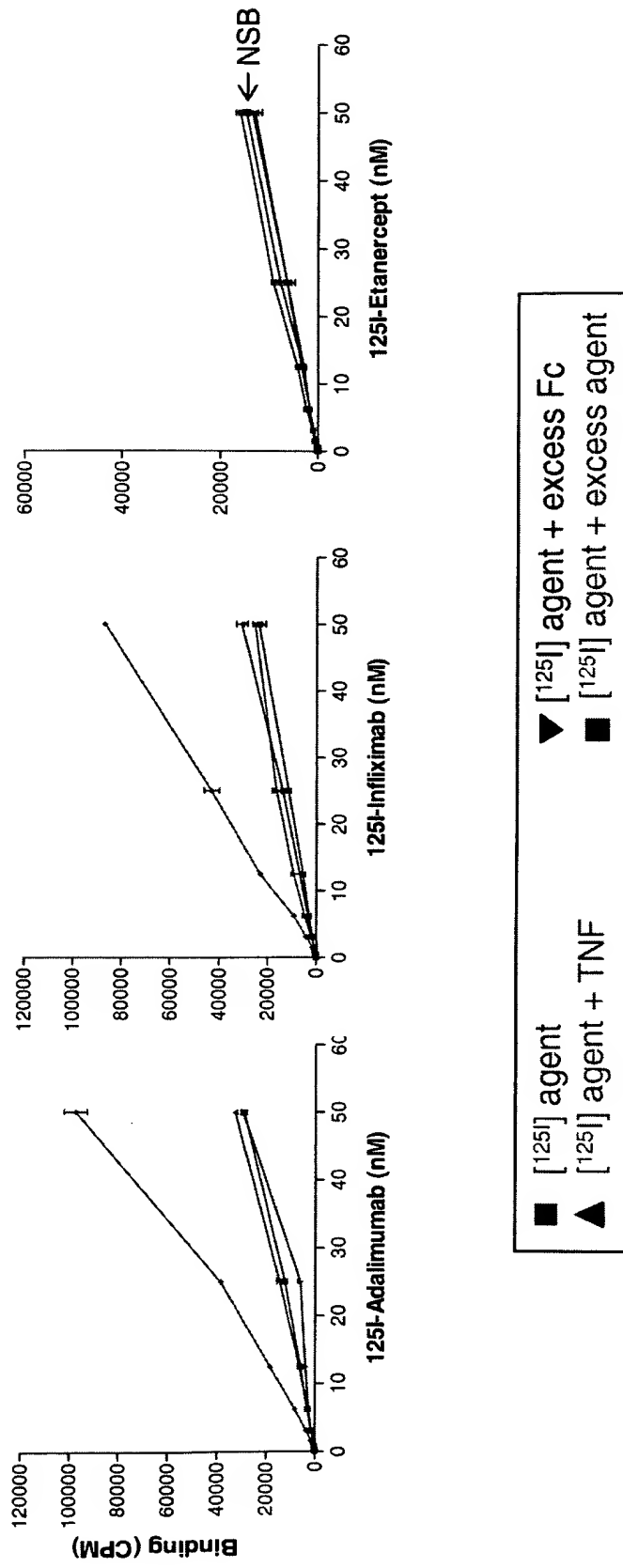


★ Precipitation lines

Ouchterlony (double diffusion) analysis of TNF antagonists



Fc γ R Binding Analysis of TNF Antagonists



C1q Binding Analysis of TNF Antagonists

AFFINITY PRECIPITATION OF ENZYMES

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1. Introduction

Bifunctional nucleotide derivatives, i.e., nucleotides connected by a spacer, such as AMP-AMP or the heterofunctional compound AMP-ATP [1], have earlier been prepared with the objective of using them primarily as affinity chromatography ligands. It occurred to us that compounds of this type might function as precipitating agents for enzymes. In particular, dimeric NAD-derivatives should be useful since NAD has affinity for a large number of enzymes. Further, these derivatives would allow utilization of the principles of ternary complex formation, which increases the interaction and ensures a high degree of specificity [2]. Such affinity precipitation of enzymes should not only provide a new tool in the analysis and purification of enzymes (dehydrogenases), but also be useful in morphologic and topographic studies of dehydrogenases in analogy to studies using bis-biotinyl diamines and avidin [3].

This paper describes the preparation of a bifunctional NAD compound, N_2, N_2' -adipodihydrazido-bis-(N^6 -carbonylmethyl-NAD) (Bis-NAD), and its properties as a complexing/precipitating agent for the tetrameric enzyme lactate dehydrogenase (LDH).

2. Materials and methods

2.1. Materials

LDH (beef heart, type III, in $(\text{NH}_4)_2\text{SO}_4$, 550 U/mg), adipic acid dihydrazide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were

obtained from Sigma, St. Louis, MO. Before use the enzyme was dialyzed overnight against 0.05 M sodium phosphate buffer (pH 7.5) and freed from insoluble matter by centrifugation. N^6 -Carboxymethyl-NAD was prepared as in [4]. All other reagents were of analytical grade and obtained from commercial sources.

2.2. Synthesis of N_2, N_2' -adipodihydrazido-bis-(N^6 -carbonylmethyl-NAD) (Bis-NAD)

Adipic acid dihydrazide dihydrochloride (105 mg, 0.60 mmol) and N^6 -carboxymethyl-NAD (900 mg, 1 mmol) were dissolved in 10 ml water (15°C) and pH adjusted to 4.0 with 1 M HCl. The condensing agent, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, was added as a 1 M aqueous solution (0°C) in 0.1 ml portions. After 30 min by which time 15 portions (1.5 mmol) had been added, thin-layer chromatography indicated that most of the N^6 -carboxymethyl-NAD had been converted and the reaction was terminated. The reaction mixture was diluted to 1.0 l and adjusted to pH 8.0 with NH_4OH . The solution was applied to a column with cellulose anion exchanger (Whatman DE-52, 2.5×80 cm), successively equilibrated with 1 M NH_4HCO_3 (pH 8.0) and water. The column was washed with water and the nucleotide was then eluted with a 4 l ammonium bicarbonate gradient from 0–0.25 M.

The desired product, Bis-NAD, was eluted between 1.2 l and 1.6 l. Lyophilization gave 400 mg of a white product which was homogeneous as judged from thin-layer chromatography. R_F values on silica gel (Merck, Darmstadt) developed in 0.5 M ammonium acetate : ethanol = 2.5 were: N^6 -carboxymethyl-NAD = 0.22; Bis-NAD = 0.05; and the monosubstituted derivative

N_2 -adipodihydrazido- N^6 -carbonylmethyl-NAD = 0.19. Based on ultraviolet spectra and phosphate analysis the ϵ -value at 266 nm in neutral aqueous solution for Bis-NAD was $21\,400\text{ M}^{-1}\text{ cm}^{-1}$. The yield based on N^6 -carboxymethyl-NAD was calculated as 44%.

2.3. Affinity precipitation of LDH (standard procedure)

The precipitation procedure was carried out at $0-6^\circ\text{C}$ in the following way: To 1.5 ml dialyzed LDH (1.1 mg/ml) in 0.05 M sodium phosphate buffer (pH 7.5) was added 0.25 ml 0.12 mM Bis-NAD (water) followed by 0.25 ml 0.8 M sodium pyruvate (water). After gentle mixing in a test tube the solution was allowed to stand. Within 30 min, a heavy precipitate began to form and after 16 h (overnight) the precipitate was isolated by centrifugation.

The amount of LDH in the precipitate and the supernatant fluid was determined by the Lowry procedure [5] and from activity measurements. LDH activity was determined by following the oxidation of NADH at 340 nm. The assay medium consisted of 1 mM sodium pyruvate and 0.30 mM NADH in 0.05 M phosphate buffer (pH 7.5). The assay was initiated by adding a suitably diluted enzyme solution (to give $\Delta A_{340} < 0.1/\text{min}$). Some enzyme inhibition due to ternary complex formation with Bis-NAD and pyruvate was sometimes observed, but was corrected for by references.

2.4. Affinity precipitation in agarose gels

Agarose gels (0.8%) were cast on microscope glass slides and wells were punched out with a die. In double diffusion experiments [6] the agarose gel contained 0.3 M sodium pyruvate and 0.05 M sodium phosphate buffer (pH 7.5); the center well contained 15 μl LDH solution (5–50 μg enzyme) and the peripheral wells contained 7 μl reagent (Bis-NAD, or NAD or buffer). After diffusion for 1.5–16 h in a moist chamber at room temperature the precipitated protein was stained with Amido black [6].

In single radial diffusion experiments [6] the agarose was cast in the presence of pyruvate and Bis-NAD when LDH was to be quantified and in the presence of pyruvate and LDH when Bis-NAD was to be quantified.

3. Results and discussion

3.1. Structural assignment

When designing the preparation of bifunctional NAD compounds, principally equivalent to the one depicted in fig.1, i.e., compounds built by connecting two NAD entities symmetrically by a spacer, it appeared reasonable to use the available compounds N^6 -carboxymethyl-NAD [4] or N^6 -[N -(6-aminohexyl)-carbamoylmethyl]-NAD [4]. A direct condensation of these two NAD analogs by a carbodiimide proved unsatisfactory since the yield was very low owing to side reactions [1]. Other methods based on condensation of two N^6 -[N -(6-aminohexyl)carbamoylmethyl]-NAD with reactive diimidoesters (adipimide) or with very reactive diacid dichlorides (adipic acid dichloride) were feasible, although the exact procedure when mixing reactants was critical and great care had to be exercised if a satisfactory result was to be expected. In addition, the connecting spacers in these cases were considered unnecessarily long and an alternative linking procedure based on N^6 -carboxymethyl derivatives of NAD was therefore tried. N^6 -Carboxymethyl-NAD was thus condensed with adipic acid dihydrazide in a carbodiimide-mediated reaction, giving a bis-nucleotide with a spacer of moderate length (fig.1). The distance between the exocyclic nitrogen atoms of the two adenines is $\sim 17\text{ \AA}$ (measured from an extended space-filling model), a distance that should allow easy and simultaneous interaction with active sites of two dehydrogenase molecules, provided the sites are not too deeply buried. The spacer is comparatively hydrophilic, which is preferable because it reduces the risk of non-specific hydrophobic interactions.

The condensation reaction described in section 2 proceeds very smoothly at pH 4.0, due to the favourable pK_a values of the reactants (2.5–3.0 for

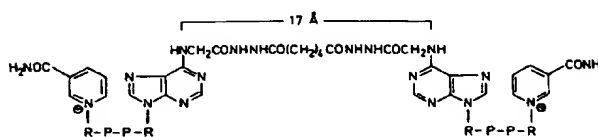


Fig.1. Bis-NAD = N_2, N_2' -adipodihydrazido-bis-(N^6 -carbonylmethyl-NAD).

*N*⁶-carboxymethyl-NAD and 4.9 for adipic acid dihydrazide) which also allow for sufficient buffering to make external pH-control virtually unnecessary.

The structure assigned to Bis-NAD is based on several facts. The method of synthesis, a carbodiimide mediated condensation of *N*⁶-carboxymethyl-NAD and adipic acid dihydrazide, should yield only two new compounds (besides breakdown products), namely adipic acid dihydrazide mono- and disubstituted with NAD. In agreement herewith, an ion-exchange chromatography of the crude reaction product gave three major peaks, the last of which to emerge was unreacted *N*⁶-carboxymethyl-NAD. The other two peaks were assigned to be the mono-substituted compound, *N*₂-adipodihydrazido-*N*⁶-carbonylmethyl-NAD (the first peak; yield ~10%) and Bis-NAD (the second peak; yield ~40%). In a separate verifying experiment the condensation was carried out with a 20-fold excess of adipic acid dihydrazide and, as expected, only one product was formed, namely the monosubstituted compound. The monosubstituted compound gave positive reaction with trinitrobenzene sulfonic acid reagent [7], whereas Bis-NAD did not, proving that the latter compound lacked the free hydrazide group.

Comparison of 100 MHz proton NMR spectra of *N*⁶-carboxymethyl-NAD, *N*₂-adipodihydrazido-*N*⁶-carbonylmethyl-NAD and Bis-NAD also confirmed the structures assumed. The two latter compounds thus gave two new signals corresponding to the protons of the four adjacent methylene groups of the spacer. Besides physicochemical tests, the Bis-NAD was shown to act as a coenzyme with LDH.

3.2. Affinity precipitation, basic properties

Initial experiments showed that LDH could be precipitated from a solution containing equivalent amounts of Bis-NAD (i.e., 1 NAD/enzyme subunit). The explanation to the precipitation event is believed to be rather straightforward. Molecules of LDH, Bis-NAD and pyruvate form strong dead-end ternary complexes and since Bis-NAD can interact with two LDH molecules and since LDH is a tetrameric enzyme it can easily be understood that large aggregates will form. When these aggregates have grown sufficiently large, they turn insoluble and precipitate out.

In order to obtain an optimal 'yield' of precipitation several parameters were varied. The pH value

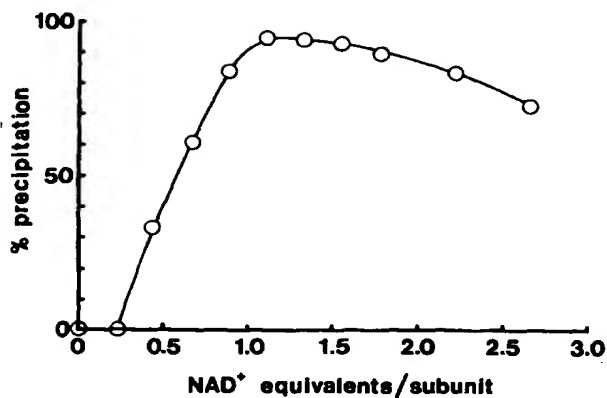


Fig.2. The efficiency of the affinity precipitation as function of the NAD-equiv./subunit ratio. LDH (20 μ N) dissolved in 0.05 M phosphate buffer (pH 7.5) 0.1 M with respect to pyruvate was treated with varying amounts of Bis-NAD in small test tubes. Total volume was 0.4 ml. The mixture was kept at 4°C overnight and the precipitated LDH centrifuged down. The amount of precipitated LDH was measured directly by the Lowry method or calculated from the LDH activity of the supernatant.

did not appreciably affect the precipitation in the region pH 6.5–8.0 (phosphate buffer). The concentration of pyruvate was not critical if well above the mM range. Therefore 0.1 M pyruvate was routinely employed.

The correct stoichiometry of reactants (Bis-NAD/enzyme) in the affinity precipitation process is obviously important. Figure 2 gives the degree of precipitation as a function of the ratio between NAD-equivalents and enzyme subunits. It is evident that maximum precipitation occurs near equimolarity of enzyme subunits and NAD. When the ratio between coenzyme equivalents and enzyme subunits is lower than unity, the precipitation yield is low, e.g., a ratio of 0.3 gives hardly any precipitation. On the other hand, if the ratio is above unity, the precipitation yield is not so markedly affected, a ratio of 2.5, for example, still giving a precipitation efficiency of 75%.

To confirm these results an additional experiment was carried out in which the composition of the precipitate was determined. For this purpose a precipitate prepared by the standard procedure was centrifuged and treated with urea to obtain a clear

solution. The solution was analyzed in ultraviolet light (266 nm and 290 nm) and the content of nucleotide and enzyme calculated. It turned out that the composition of the precipitate corresponded well to the ratio at which the precipitation was best, i.e., 1.1 NAD equiv./LDH subunit.

To obtain macromolecular aggregates, a minimum of 2 (average) subunits/LDH molecule must be engaged in complexes, and this minimum corresponds to a ratio of 0.5; when all subunits are engaged the ratio would be 1.0, a value close to the observed value of 1.1. This reasoning is, of course, valid only with the assumption that the cofactor, pyruvate and enzyme form a firm complex. It is also assumed that Bis-NAD does not participate in intramolecular crosslinking, an unlikely situation since the 17 Å spacer (fig.1) would be too short to cover the distance between two cofactor binding sites within the same molecule [8].

3.3. Redissolution of affinity precipitated dehydrogenase

In some applications of affinity precipitation, e.g., purification of enzymes, it would be necessary to remove pyruvate and Bis-NAD as a final step, and in such a way as to preserve activity. Table 1 illustrates the feasibility of regenerating the free enzyme from the precipitate by gel filtration. In order to discriminate between losses of activity due

to the affinity precipitation process and losses due to the regeneration, several references were included. Gel filtration on Sephadex G-50 gave a recovery of ~85%, the 15% loss of activity being caused both by the precipitation and the gel filtration, as judged from the references. Prior to the gel filtration the precipitate had to be dissolved and to this end NADH was added to 10 mM. The precipitate dissolved within 1 min, the mechanism behind the phenomenon obviously being that NADH forms a strong complex with LDH ($K_{\text{diss}} \sim 1 \mu\text{M}$, NADH is present in high concentration) and thus efficiently competes for the active sites. Also, the simultaneous presence of NADH and LDH would consume the comparatively small amount of pyruvate present in the precipitate. Another competitive ligand, AMP (10 mM) was also tried, but was less efficient in solubilizing the precipitate in accordance with its higher dissociation constant, $K_{\text{diss}} \sim 1 \text{ mM}$. Summarizing, it is feasible by simple means to regenerate affinity precipitated material.

3.4. Affinity precipitation in gels

Affinity precipitation of enzymes resembles, at least superficially, immunoprecipitation, i.e., the aggregation of antibodies and antigens. In a set of experiments we exploited this and adopted the techniques commonly used in immunodiffusion

Table 1
Recovery of affinity precipitated LDH

Sample reference	Additions in the precipitation step	Treatment in the recovery step	Recovery %
Sample	Pyruvate + Bis-NAD	NADH; G-50	85
Reference 1	Pyruvate + NAD	NADH; G-50	92
Reference 2	None	NADH; G-50	92
Reference 3	None	None	100

Samples and references contained 25 μN LDH in 0.05 M phosphate buffer (pH 7.5). In the precipitation step pyruvate (0.1 M) and Bis-NAD (30 μN) or NAD (30 μN) were added as indicated. The total volume was 1.20 ml. After 16 h the precipitate (formed in the samples only) was centrifuged down and the supernatant discarded. In the recovery step the LDH in samples and references was freed from pyruvate and nucleotide by gel filtration (Sephadex G-50, 1.5 \times 30 cm; 1 ml/min). Before application on the G-50 column the sample and the reference were steeped in 10 mM NADH (in order to dissolve the precipitated protein in the sample). The activity of the LDH after the various treatments is given relative to that of reference 4 (100%)

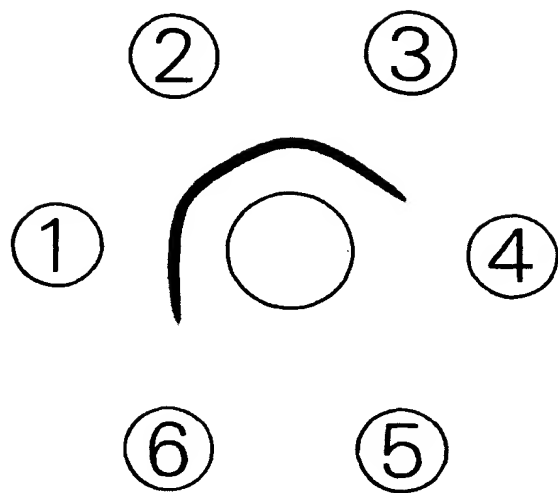


Fig.3. Diffusion-precipitation in agarose gel. The center well contained 30 μ g lactate dehydrogenase in 15 μ l buffer. The peripheral wells contained 7 μ l reagents: wells 1–3, 1.5 nmol Bis-NAD; well 5, 3.0 nmol NAD; wells 4, 6, buffer only.

experiments. Figure 3 shows the result of a double diffusion experiment (Ouchterlony test [6]) in agarose-containing pyruvate, where the center well contained enzyme, the peripheral wells Bis-NAD or NAD or buffer. A precipitation band was observed only where LDH and Bis-NAD had been allowed to diffuse towards each other. The figure shows the situation after 1.5 h diffusion. Prolonged diffusion for 16 h gave approximately the same pattern. The single radial diffusion technique (Mancini method [6]) was briefly evaluated with respect to its ability to quantify lactate dehydrogenase and/or Bis-NAD. The agarose gel in this case was cast in the presence of pyruvate and Bis-NAD (or lactate dehydrogenase) and the linearly-arranged wells were filled with different amounts of lactate dehydrogenase (or Bis-NAD). Precipitation rings were formed around the wells; the diameters being approximately proportional to the amount of lactate dehydrogenase (or Bis-NAD) present in the well.

4. Conclusion

Affinity precipitation of enzymes has been

exemplified here with lactate dehydrogenase and Bis-NAD (+ pyruvate) and is likely to be applicable also to other NAD-dependent dehydrogenases although preliminary experiments with liver alcohol dehydrogenase and the Bis-NAD used in this study (+ pyrazol) indicate that at least for alcohol dehydrogenase a longer spacer is required. Affinity precipitation should be applicable also to enzymes/proteins other than dehydrogenases, provided that suitable bifunctional ligands are available. In cases in which the interaction between the bifunctional ligand and the enzyme is not sufficiently strong, formation of ternary complexes could be tried as exemplified here. Thus, besides dead-end complex formation also other ternary complexes could be utilized, for instance complexes with coenzyme and inhibitor and complexes with coenzyme-substrate adducts [9]. Alternatively, a careful addition of salts, e.g., ammonium sulphate or solvents, e.g., polyethylene glycol might enhance the precipitation without impairing the specificity.

Affinity precipitation of enzymes using bis-nucleotides of varying spacer length may be useful in topographic studies of enzymes, e.g., in the determination of the depth of an active site/ligand binding site. Also information concerning the spatial arrangements of subunits in an oligomeric enzyme might be obtained from precipitated aggregates by using electron microscopy analogous to the study of complexes between avidin and bisbiotinyl diamines [3]. In certain cases enzyme purification procedures may benefit from the principle, e.g., when conventional affinity chromatography is less satisfactory because of sterically-impaired interaction between the enzyme and its immobilized ligand. Further, the method of diffusion-precipitation in gels might find, for example, clinical applications. The procedure could, for instance, be used for detecting abnormal levels of enzymes and other proteins found in serum; compared with the immunodiffusion technique an obvious advantage of this method is that antibodies, which might be difficult to raise, would not be needed.

Finally, it deserves mentioning that bifunctional ligands (including those interacting with effector sites) may prove useful as agents permitting precipitation/immobilization of biomolecules in a reversible manner.

Acknowledgement

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I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail, in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Dated: December 9, 2004

Signature

Sharon M. Sirtich

Docket No.: 01017/40451C

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Brockhaus et al.

Application No.: 08/444,791

Group Art Unit: 1644

Filed: May 19, 1995

Examiner: R. Schwadron, Ph.D.

For: Human TNF Receptor

DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. WERNER LESSLAUER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Dr. Werner Lesslauer, do hereby declare and state as follows:

1. I am a co-inventor of the invention claimed in the above-referenced application. I am familiar with the contents of the above-identified U.S. patent application and I am providing this declaration to make available to the Examiner additional data relevant to the invention claimed.

2. Attached hereto as Exhibit A is an affidavit in German that I supplied to the European Patent Office regarding counterpart European patent application no. 99100703.0 (European patent publication no. EP 0 939121 B1).

3. Attached hereto as Exhibit B is an English translation of the affidavit. I hereby confirm my belief in the truth of the statements in this English translation for submission to the U.S. Patent and Trademark Office in the above-identified application.

4. The experiments described in Exhibits A and B compared the activity of a recombinant soluble fragment of the human p75 TNF receptor (p75sTNFR) to the activity of a recombinant immunoglobulin (Ig) fusion protein of p75sTNFR referred to as "p75sTNFR/IgG." The recombinant Ig fusion protein consists of the soluble extracellular domain of the 75 kD TNF receptor, the cloning of which is described in the specification in Example 8, fused to a fragment of the heavy chain of a human IgG3 molecule which is missing the CH1 domain and contains the hinge, CH2 and CH3 domains. This p75 fusion protein is described, *inter alia*, at page 11, lines 1-14.

Application No.: 08/444,791
Declaration of Werner Lesslauer

5. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. § 1001), and may jeopardize the validity of the application or any patent issuing thereon.

Date

December 3, 2004

Werner Lesslauer

Dr. Werner Lesslauer

Eidesstattliche Erklärung

Ich, Werner Lesslauer, Dr. med., Dr. phil. nat., Privatdozent, zur Zeit Gastprofessor der Yale University School of Medicine, Dept. Epidemiology & Public Health and Immunobiology, 60 College Street, New Haven, CT 06520-8034, USA, mache die folgende eidesstattliche Erklärung.

Vom Jahr 1987 bis Ende Juni 1999 war ich in den Biologie-Abteilungen der Zentralen Forschungseinheit und der Forschungsabteilung Zentrales Nervensystem der Hoffmann - La Roche AG in Basel (Schweiz) tätig, zuletzt als wissenschaftlicher Experte verantwortlich für die Leitung verschiedener Forschungsgruppen in den Bereichen der Protein-, Zell- und Molekularbiologie. Seit Anfang September 1999 bin ich als Gastprofessor an der Universität Yale tätig. Meine gegenwärtigen Forschungsprojekte betreffen von pro-inflammatorischen Cytokinen wie zum Beispiel TNF α oder Lymphotoxin (gemeinsam als "TNF" bezeichnet) vermittelte interzelluläre Kommunikation, die von den zellulären Rezeptoren dieser Cytokine aktivierten intrazellulären Signaltransduktions-Mechanismen, und die durch solche Prozesse im Rahmen von Entzündungsphänomenen ausgelöste organoide Transformation von tertiären lymphoiden Geweben. Im weiteren befasste ich mich mit der Rolle von Cytokin-aktivierter Signaltransduktion in kognitiven Funktionen. Diese Arbeiten stellen somit eine Weiterführung der bei Hoffmann-La Roche verfolgten wissenschaftlichen Interessen dar. Ein Teil meines Verantwortungsbereichs bei der Hoffmann - La Roche AG umfasste die Entwicklung von Verfahren zur rekombinanten Expression und zum Reinigen und Testen von Proteinen, wie beispielsweise den löslichen TNF - Rezeptoren ("sTNFR") und p75TNF - Rezeptor - Immunglobulin - Fusionsproteinen ("p75sTNFR/IgG"). Diese Rezeptor-Fusionsproteine wurden durch die Fusion der löslichen extrazellulären Domäne des p75TNF - Rezeptors, p75sTNFR, die selbst TNF bindet, mit einem Fragment der schweren Kette eines humanen IgG - Moleküles, das praktisch dem Fc-Teil entspricht, mit biotechnologischen Verfahren konstruiert.

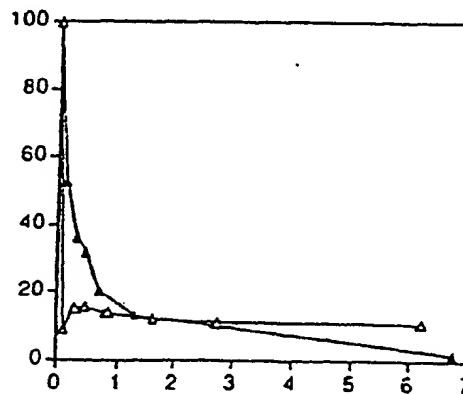
Weiterhin bin ich einer der Miterfinder der vorliegenden Europäischen Patentanmeldung mit der Anmeldenummer 99.100703.0, die solche p75sTNFR/IgG beansprucht. Ich war sowohl an der Erfindung, der Herstellung und dem Testen dieser Fusionsproteine beteiligt.

Gegenstand der vorliegenden eidesstattlichen Erklärung sind die im Vergleich zu der löslichen extracellulären Domäne des p75TNF - Rezeptors p75sTNFR überraschenden Eigenschaften von p75sTNFR/IgG. Zum Zeitpunkt, in dem p75sTNFR/IgG erstmals konstruiert, exprimiert und getestet wurde, gab es Vorstellungen über die räumliche Struktur von TNF α . In dem betreffenden Proteinkristall lag TNF α als Trimer vor und es wurde vermutet, dass dies nicht allein eine Folge der Kristallisierung war, sondern dass das TNF α -Trimer auch die biologisch aktive Form ist. Die räumliche Geometrie der Rezeptor-Bindungsstelle war jedoch unbekannt. Es wäre durchaus möglich gewesen, dass die Fusion mit IgG - Fragmenten ein räumliches Gebilde geschaffen hätte, das wohl TNF-Rezeptorsequenzen enthalten hätte, das aber wegen seiner räumlichen Struktur TNF α überhaupt nicht binden konnte.

Die rasche Elimination und daher kurze Halbwertszeit von p75sTNFR in vivo machte jedoch eine Vergrößerung des Moleküls unerlässlich. Es ist nicht auszuschliessen, dass man sogar eine gewisse Einbusse an Bindungsaktivität in Kauf genommen hätte, um nur eine längere Halbwertszeit und Bioverfügbarkeit zu erreichen. Ueberraschend zeigte das Fusionskonstrukt

jedoch sogar eine sehr gute Bindungsaktivität. Zudem fand sich eine unerwartet höhere kinetische Stabilität, und eine überraschend bessere Inhibierung der Wirkung von TNF α in biologischen Zellkultur-Testen.

Die höhere kinetische Stabilität von p75sTNFR/IgG lässt sich durch den folgenden Versuch I (siehe Figur) veranschaulichen: p75sTNFR/IgG und p75sTNFR werden in separaten Reaktionsgefäßen mit radioaktivem TNF α inkubiert, sodass die jeweiligen molekularen Spezies im Komplex mit markiertem TNF α vorliegen. Diese Komplexe werden sodann in neue Lösungen überführt, die einen Ueberschuss an unmarkiertem TNF α enthalten. Wie allgemein in jeder Bindungsstudie wurde auch hier gefunden, dass TNF α mit einer für den jeweiligen Reaktionspartner spezifischen Kinetik an p75sTNFR/IgG und p75sTNFR andockt und wieder dissoziiert. In zeitabhängiger Weise wurde nun die Austauschrate von kaltem TNF α mit dem jeweils an p75sTNFR/IgG und p75sTNFR gebundenen markierten TNF α bestimmt. Eine genaue Beschreibung der experimentellen Technik dieses Versuches wird im Annex gegeben. Die Ergebnisse dieser Experimente sind in der folgenden Figur dargestellt. In dieser ist auf der waagrechten Achse die Zeit in Stunden und auf der senkrechten Achse der Anteil an spezifisch gebundenen radioaktiv markiertem TNF α in % angegeben. Nichtgefüllte Dreiecke stehen für p75sTNFR und gefüllte Dreiecke stehen für p75sTNFR/IgG.



Dieser Figur kann man klar entnehmen, dass am von der Versuchsanordnung her frühest möglichen ersten Messzeitpunkt, d.h. nach etwa sechs Minuten, das gesamte an p75sTNFR gebundene TNF α bereits vollständig ausgetauscht worden war. Hingegen waren bei p75sTNFR/IgG zu diesem Zeitpunkt erst etwa die Hälfte der markierten TNF α Moleküle ausgetauscht worden. Dies bedeutet, dass TNF α mit einer wesentlich langsameren Kinetik von p75sTNFR/IgG dissoziiert als von p75sTNFR. Damit wird die Wirkung von TNF α durch

p75sTNFR/IgG wesentlich besser neutralisiert als durch p75sTNFR, da das freigewordene TNF α wieder biologische Aktivität entfalten kann. Diese Eigenschaft lässt das p75sTNFR/IgG Fusionskonstrukt ganz unabhängig von der durch die Vergrößerung des Moleküles bedingten langsameren Elimination in vivo als potenteres pharmakologisches Agens erscheinen.

Diese unvorhersagbare Eigenschaft korreliert auch mit einer unerwartet besseren Inhibierung der Wirkung von TNF durch p75sTNFR/IgG gegenüber p75sTNFR, wie der folgende Versuch II (siehe Tabelle) verdeutlicht. Dabei handelt es sich um einen Versuch in Zellkultur mit weissen, sog. mononukleären Blutzellen, die aus humanem Blut isoliert worden waren. Diese Zellen lassen sich in Kultur durch Behandlung mit mitogenen Substanzen zur Proliferation stimulieren, die dadurch zu Stande kommt, dass einzelne Zellgruppen in der Kultur durch die Mitogen-Behandlung ausgelöst sekundär Wachstumsfaktoren produzieren und in das Kulturmedium abgeben. Der bekannteste dieser Wachstumsfaktoren ist das wohlbekannte Interleukin-2. Daneben hat es sich gerade durch Untersuchungen, die durch die Verfügbarkeit von Reagentien wie p75sTNFR/IgG und p75sTNFR ermöglicht wurden, gezeigt, dass unter anderem auch TNF in den späteren Phasen solcher Kulturen eine zellwachstumsfördernde Aktivität entfaltet. Die Eigenschaft von p75sTNFR/IgG und von p75sTNFR, TNF zu binden und zu neutralisieren, erlaubt nun, diese proliferative Aktivität von TNF zu inhibieren. Das Ergebnis eines derartigen Versuchs ist in der untenstehenden Tabelle festgehalten. In diesem Versuch wurde die Zellproliferation durch den Einbau der radioaktiv markierten Vorstufe Thymidin in die zelluläre DNA gemessen (siehe Annex).

Verwendete Konstrukte

Inhibierung des Einbaus von
Deuterium - Thymidin (Tag 7)

p75sTNFR	68 %
p75sTNFR/IgG	86 %

Aus dieser Tabelle ist klar ersichtlich, dass das Fusionsprotein p75sTNFR/IgG gegenüber der löslichen extrazellulären Domäne p75sTNFR eine ueberraschend bessere Neutralisierung der TNF Aktivität, d.h. der Proliferation der Blut- Zellen in Kultur, bewirkt.

Eine derart potentere neutralisierende Wirkung ist in pathologischen Zuständen, die durch zu starke TNF α Freisetzung mitverursacht werden, sehr erwünscht. Es ist hier wichtig, daran zu erinnern, dass TNF α zwar in vielen pathologischen Zuständen ein wichtiger Faktor der Wirtsabwehr ist und damit für den Organismus eine wichtige positive Funktion hat. TNF α hat jedoch ein Janus-Gesicht, und entfaltet in anderen Situationen - sei es durch zu starke Expression, Expression am falschen Ort, oder zur falschen Zeit - krankmachende Wirkungen. Da man bereits zum Zeitpunkt der vorliegenden Anmeldung annahm, dass bei einer Reihe von Krankheiten, wie beispielsweise der rheumathoiden Arthritis, TNF α in der Entstehung der Entzündung und in der Gewebs-Zerstörung in den Gelenken eine Rolle als krankmachender

Mediator spielt, sollten Substanzen welche die Wirkung von TNF α inhibieren auch bei der Behandlung solcher Krankheiten als pharmazeutisch wirksame Substanzen einsetzbar sein. Diese Gedankengänge haben später durch die erfolgreiche Einführung eines p75sTNFR-IgG Präparates in die Therapie der rheumatoiden Arthritis ihre volle Bestätigung gefunden.

ANNEX

Versuch I:

Man inkubiert 1.4 μ g/ml des p75sTNFR/IgG bzw. 0.75 μ g/ml des p75sTNFR in 1 ml Phosphat- gepufferter Kochsalzlösung ("PBS" enthaltend 1% foetales Kälberserum) mit 25 ng/ml 125 I-markiertem TNF α , das in seiner Rezeptor-Bindung von unmarkiertem TNF α nicht zu unterscheiden war. Zum Zeitpunkt Null setzt man dann einen 1000-fachen Ueberschuss nichtmarkiertes TNF α dazu und entnimmt zu verschiedenen Zeitpunkten jeweils kleine Proben von 60 μ l. Diese Proben gibt man in Millipore 0.22 μ MC Filtereinheiten die bereits 20 μ l einer 50%igen Suspension von 'Protein G Sepharose 4 Fast Flow Beads' ('Sepharose-Kugeln') in PBS mit 1% fötalem Kälberserum enthalten. Damit p75sTNFR/IgG bzw. p75sTNFR an die Sepharose Kugel binden können, wurden diese mit einem gegen den TNF-Rezeptor gerichteten Antikörper vorbeschichtet (1 mg Antikörper/ ml Sepharose-Kugeln). Nach Inkubation während 4 min. unter Schütteln wurden die Filtrationseinheiten zentrifugiert (13000 rpm, 30 sek.), und damit das ungebundene TNF α abgetrennt, während das an p75sTNFR-IgG bzw. p75sTNFR gebundene TNF α auf den Sepharose-Kugeln haften blieb. Damit wurde es möglich, die am jeweiligen Zeitpunkt noch am p75sTNFR-IgG und p75sTNFR gebundene Menge von radioaktiv markiertem TNF α zu messen. Nicht-spezifische Bindung wurde in derselben Weise in Abwesenheit von p75sTNFR/IgG bzw. p75sTNFR bestimmt. 100%ige Bindung wurde in Abwesenheit von nichtmarkiertem TNF α bestimmt. Die Versuche wurden bei 25°C durchgeführt.

Versuch II

Mononukleäre Zellen wurden aus frischem venösen Human- Blut von gesunden Spendern mittels eines Ficoll Paque- Dichtegradienten (Pharmacia, Uppsala, Schweden) in einem Zitratpuffer isoliert. Diese weissen Blut - Zellen wurden zweimal mit einer Phosphat- gepufferter Kochsalzlösung gewaschen und bei einer Dichte von 1.0×10^6 Zellen/ml in RPMI 1640 Kulturmedium, das mit 10%igem hitzeinaktivierten fötalem Kälberserum, 100 Einheiten/ml Penizillin, 100 μ g/ml Streptomycin und 2 mM Glutamin supplementiert worden war, kultiviert. Für den Proliferationstest wurden die Zellen in Flachbodenmikrotiterplatten (NUNC/NON 1-67008; Roskilde, Dänemark) in 100 μ l Medium kultiviert. Die Zellen wurden, mit Phytohaemagglutinin (Wellcome, Dartford, England) bei zuvor bestimmten optimalen Konzentrationen im Bereich von 0.5 bis 1.5 μ g/ml stimuliert. Zum Startzeitpunkt der Kultur (Tag 0) wurde p75sTNFR/IgG bzw p75sTNFR bis zu eine Konzentration von 10 μ g/ml zugegeben. Das Kulturmedium wurde nach 3, 4 und 6 Tagen aufgefrischt. Die

Exhibit A

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Zellproliferation wurde nach 7 Tagen gemessen, wobei den Kulturen sechs Stunden vor dem Ernten mit einem LKB Zellmarker $1\mu\text{Ci/Kultur}$ Methyl- 3H -Thymidin (1mCi/ml Amersham, Buckinghamshire, England) zugesetzt wurde. Die in die Zellen eingebaute Radioaktivität wurde in einem Betaplatten-Flüssig Szintillationszähler (Pharmacia, Uppsala, Schweden) gemessen. Die dargestellten Werte stellen den Mittelwert von drei Kulturen dar.

New Haven, 8. Oktober 2001

W. Leuker

Exhibit B

Affidavit

I, Werner Lesslauer M.D., Ph.D., Private Lecturer, presently Visiting Professor at the Yale University School of Medicine, Dept. Epidemiology & Public Health and Immunobiology, 60 College Street, New Haven, CT 06520-8034, USA, hereby file an affidavit in lieu of an oath:

From 1987 to the end of June of 1999, I was working in the Biology Departments of the Central Research Unit and the Research Department, Central Nervous System, of Hoffmann-LaRoche AG in Basel (Switzerland); toward the end of my activity, I worked as scientific expert and was responsible for the management of different research groups in the fields of protein, cell and molecular biology. At the beginning of September 1999, I began my work as a Visiting Professor at Yale University. My current research projects relate to the intercellular communication mediated by pro-inflammatory cytokines, such as TNF α or lymphotoxin (jointly called "TNF"), the intracellular signal transduction mechanisms activated by the cellular receptor of these cytokines, and the organoid transformation of tertiary lymphoid tissue triggered by such processes in the context of inflammatory phenomena. In addition, I am also working on the role of cytokine-activated signal transduction in cognitive functions. Thus, my current research extends the scientific interests I pursued at Hoffmann-LaRoche. As part of my responsibilities at Hoffmann-LaRoche AG, I worked on the development of methods for the recombinant expression and for the purification and testing of proteins, such as the soluble TNF receptors ("sTNFR") and p75TNF receptor immunoglobulin fusion proteins ("p75sTNFR/IgG"). These receptor fusion proteins were constructed by means of the fusion of the soluble extracellular domain of the p75TNF receptor, p75sTNFR, which itself bind TNF, and a fragment of the heavy chain of a human IgG molecule which practically corresponds to the Fc portion, using biotechnological methods.

I am also one of the co-inventors of the present European Patent Application with the Application Number 99.100703.0 which claims such p75sTNFR / IgGs. I participated both in the invention and in the production and testing of these fusion proteins.

The subject matter of the present affidavit in lieu of an oath concerns the properties of p75s TNFR / IgG which are surprising when comparing them to those of the soluble extracellular domain of the p75TNF receptor, p75sTNFR. At the time when p75sTNFR / IgG was first constructed, expressed, and tested, knowledge of the spatial structure of TNF α was available. In the relevant protein crystal, TNF α was present in the form of a trimer, and it was

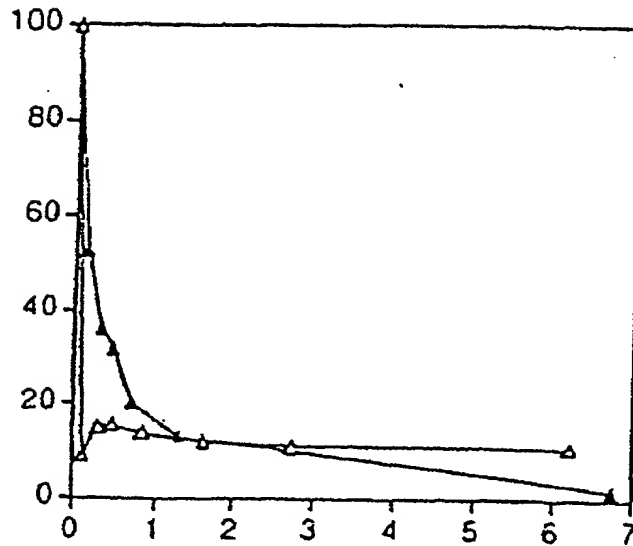
Exhibit B

hypothesized that this was not only a result of the crystallization but that instead, the TNF α trimer is the biologically active form as well. But the spatial geometry of the receptor binding site was unknown. Thus, it could have been possible that the fusion with IgG fragments created a spatial structure that would have contained TNF receptor sequences but which, due to its spatial structure, was completely unable to bind TNF α .

The rapid elimination and thus the short half-life of p75sTNFR *in vivo*, however, made it imperative to enlarge the molecule. It cannot be excluded that there might even have been a willingness to accept a certain decrease of the binding activity only to obtain a longer half-life and greater bioavailability. Surprisingly, however, the fusion construct obtained even had an excellent binding activity. In addition, an unexpectedly higher kinetic stability and a surprisingly improved inhibition of the effect of TNF in biological cell culture tests were discovered as well.

The higher kinetic stability of p75sTNFR / IgG can be illustrated on the basis of the following experiment I (see figure): In separate reaction vessels, p75sTNFR / IgG and p75s TNFR are incubated with radioactively labeled TNF α so that the respective molecular species are present in the complex with labeled TNF α . These complexes are subsequently transferred into new solutions which contain an excess of unlabeled TNF α . As is generally the case in any binding study, it was found here as well that TNF α binds to and dissociates itself from p75sTNFR / IgG and p75sTNFR with kinetics specific to the respective reaction participant. Next, the exchange rate of cold TNF α with the labeled TNF α bound to p75sTNFR / IgG and p75sTNFR was determined as a function of time. A detailed description of the experimental technique of this test can be found in the appendix. The results of these experiments are illustrated in the figure below. In this figure, the time in hours is plotted on the horizontal axis, and the percentage of specifically bound radioactively labeled TNF α in % is plotted on the vertical axis. Unfilled triangles stand for p75sTNFR and filled triangles stand for p75sTNFR / IgG.

Exhibit B



This figure indicates very clearly that, based on the experimental set-up, at the earliest possible first time of taking a reading, i.e., after approximately six minutes, the labeled TNF α bound to p75sTNFR had been completely exchanged. For p75sTNFR / IgG, on the other hand, at that point in time, only approximately half of the labeled TNF α molecules had been exchanged. This means that TNF α dissociated with considerably slower kinetics from p75sTNFR / IgG than it does from p75sTNFR. Thus, the effect of TNF α is considerably better neutralized by p75sTNFR / IgG than by p75sTNFR, since the liberated TNF α is able to become biologically active again. This property, quite apart from the elimination *in vivo* which is slower as a result of the enlargement of the molecule, makes the p75sTNFR / IgG fusion construct a more potent pharmacological agent.

As experiment II below (see table) illustrates, this unforeseeable property also correlates with an unexpectedly superior inhibition of the effect of TNF by p75sTNFR / IgG as compared to p75sTNFR. This test is carried out in a cell culture with white, so-called mononuclear, blood cells which had been isolated from human blood. In culture, these cells can be made to proliferate by treating them with mitogenic substances, which proliferation is propagated by the fact that individual cell groups in the culture produce growth factors which are secondarily triggered by the mitogen treatment and which are released into the culture medium. The best known of these growth factors is the well-known interleukin-2. In addition, tests which were made possible because of the availability of reagents, such as p75sTNFR / IgG and p75sTNFR, had shown that, among other things, TNF also develops a cell growth-promoting activity in the later phases of such cultures. The property of p75sTNFR / IgG and of p75sTNFR to bind and neutralize TNF makes it possible to inhibit this proliferative activity of TNF. The result of such a test is summarized in the table below. In this test, the cell proliferation was measured by

Exhibit B

incorporation of the radioactively labeled precursor thymidine into the cellular DNA (see Annex).

<u>Construct Used</u>	<u>Inhibition of the incorporated ³H-Thymidine (day 7)</u>
p75sTNFR	68%
p75sTNFR / IgG	86%

This table illustrates clearly that compared to the soluble extracellular domain p75sTNFR, the fusion protein p75sTNFR / IgG causes a surprisingly superior neutralization of the TNF activity, i.e., the proliferation of the blood cells in culture.

This more highly potent neutralizing effect is very desirable in pathological conditions that are caused by an excessively high TNF α release. In this context, it is important to keep in mind that, although in many pathological conditions TNF α is an important factor of the host's defense and thus plays an important positive role within the organism, TNF α has two faces and, in different situations, develops effects that cause disease -- either by an excessively high expression, or an expression in the wrong site or at the wrong time. Since it was assumed as early as at the time of the present application that in a number of diseases, such as rheumatoid arthritis, TNF α plays a role as a disease-causing mediator in the development of the inflammation and in the destruction of the tissue in the joints, the next step was to assume that it should be possible to use substances that inhibit the effect of TNF α as pharmaceutically effective substances in the treatment of such diseases. Later on, these hypotheses were fully corroborated when a p75sTNFR-Ig/G preparation was successfully introduced into the therapy of rheumatoid arthritis.

Appendix

Experiment I:

1.4 μ g / mL of p75sTNFR / IgG and 0.75 μ g / mL of p75sTNFR were separately incubated in 1 mL of phosphate-buffered physiological saline solution ("PBS" containing 1% fetal calf serum) with 25 ng / mL ¹²⁵I-labeled, TNF α which, with respect to its receptor binding property, was not distinguishable from unlabeled TNF α . At time zero, a 1000-fold excess of unlabeled TNF α was added, and small samples of 60 μ L were taken at different times. These samples were placed into Millipore 0.22 μ MC filter units which already contained 20 μ L of a 50% suspension of 'Protein G Sepharose 4 Fast Flow Beads' ("sepharose beads") in PBS with 1% fetal calf serum. To ensure that p75sTNFR / IgG and p75sTNFR can bind to the sepharose beads, these beads had been coated earlier with an antibody directed against the TNF receptor (1 mg of antibody / mL of sepharose beads). After an incubation time of 4 min with shaking, the filtration units were centrifuged (13000 rpm, 30 sec), thus separating the unbound TNF α , while the TNF α bound to p75sTNFR-IgG and p75s TNFR adhered to the sepharose beads. This made it possible to measure the quantity of radioactively labeled TNF α that was still bound to p75sTNFR-IgG and p75sTNFR at a given time. Nonspecific binding was determined in the same manner in the

Exhibit B

absence of p75s TNFR / IgG and p75sTNFR. 100% binding was determined in the absence of unlabeled TNF α . The experiments were carried out at 25°C.

Experiment II:

Mononuclear cells were isolated from fresh venous human blood of healthy donors by means of a Ficoll Paque density gradient separator (Pharmacia, Uppsala, Sweden) in a citrate buffer. These white blood cells were washed twice with a phosphate-buffered physiological saline solution and cultured at a density of 1.0×10^6 cells / mL in RPMI 1640 culture medium which had been supplemented with 10% heat-inactivated fetal calf serum, 100 units / mL penicillin, 100 μ g / mL streptomycin, and 2 mM glutamine. For the proliferation test, the cells were cultured in flat bottom microtiter plates (NUNCLON 1-67008; Roskilde, Denmark) in 100 μ L medium. The cells were stimulated with phytohemagglutinin (Wellcome, Dartford, England) at previously determined optimum concentrations in a range from 0.5 to 1.5 μ g / mL. At the time the culture was started (day 0), p75sTNFR / IgG and p75sTNFR, respectively, up to a concentration of 10 μ g / mL were added. The culture medium was renewed after 3, 4, and 6 days. The cell proliferation was measured after 7 days; 6 hours prior to harvesting with the LKB cell harvester, 1 μ Ci of methyl- 3 H-thymidine (1 mCi / ml, Amersham, Buckinghamshire, England) per culture was added. The radioactivity incorporated into the cells was measured in a betaplate liquid scintillation counter (Pharmacia, Uppsala, Sweden). The values recorded are the mean value of three cultures.

[handwritten:]

New Haven, October 8, 2001

[signature of W. Lesslauer]



I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: M5 Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Date: 11/10/06

Signature: *[Handwritten Signature]*

Docket No.: 9189 (A-947B)
01017/40451)

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Brockhaus et al.

Application No.: 08/444,790

Art Unit: 1646

Filed: May 19, 1995

Examiner: Z. Howard

For: HUMAN TNF RECEPTOR

THIRD DECLARATION OF DR. WERNER LESSLAUER UNDER 35 U.S.C. § 1.132

I, Dr. Werner Lesslauer, hereby declare as follows that:

1. I am a named co-inventor of the above-referenced application, which claims priority of a U.S. application filed on September 10, 1990. At the time the patent application was filed, I was a staff scientist at F. Hoffmann-LaRoche AG in Basel, Switzerland (Roche).
2. I have recently re-reviewed the text of the above-referenced application.
3. The activities described in paragraph 4 below were carried out in our laboratories at Roche at my request, and I have reviewed laboratory notebooks describing the activities described in paragraphs 4 and 5 below.
4. A DNA construct designated N227 containing DNA sequence which includes sequence encoding the signal sequence and the extracellular domain of human p75 tumor necrosis factor receptor (TNFR) was constructed on a date before September 10, 1990.
5. Amplified copies of the N227 construct were made and samples were stored in a freezer at Roche in a container labeled with the N227 designation.

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Application No.: 08/444,790

Docket No.: 01017/40451B

6. I have been informed that a sample from this container was shipped to Amgen, Inc. Seattle, WA and that Amgen, Inc. deposited an amplified copy of this DNA construct with the American Type Culture Collection on October 17, 2006 under Accession No. PTA-7942.

7. The DNA sequence within construct N227 is a DNA sequence identified in the above-referenced application at page 10, line 34.

8. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. § 1001), and may jeopardize the validity of the application or any patent issuing thereon.

CH-4125 Richard
Dated: November 9, 2006

Werner Lesslauer

Dr. Werner Lesslauer

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Brockhaus et al.

Application No.: 08/444,790

Art Unit: 1646

Filed: May 19, 1995

Examiner: Z. Howard

For: HUMAN TNF RECEPTOR

DECLARATION OF STEWART LYMAN, PH.D. UNDER 37 C.F.R 1.132

1. I received a Ph.D. in Oncology from the McArdle Laboratory for Cancer Research at the University of Wisconsin-Madison in 1984. I did postdoctoral research at the Fred Hutchinson Cancer Research Center in Seattle, and joined Immunex Corporation as a molecular biologist in 1988. I was a scientist at Immunex Corporation for 14 years, eventually becoming Director of Extramural Research. After Amgen acquired Immunex in 2002, I stayed on and worked for Amgen for three months in a transitional role. Since 2004, I have managed my own consulting business, and I am being compensated for my time consulting on this matter at my usual hourly rate. I also own 100 shares of Amgen stock. My Curriculum Vitae is attached as Exhibit A.
2. I have reviewed the above-identified application as originally filed. I have also reviewed the presently pending claims, attached as Exhibit B. I have been informed that the effective filing date of the above-identified application is September 10, 1990.
3. I have been informed that the Patent Office has rejected the claims as failing to comply with the written description requirement, in an Office Action dated

February 23, 2007. I have reviewed pages 8-13 of the Office Action and make this declaration to address the issues raised by the statements reproduced in Exhibit C.

4. I have been informed that the standard for satisfying the written description requirement is that one skilled in the art, upon reading the specification, would recognize that the inventors had possession of the invention that is claimed. In addition, I have been informed that "possession" does not necessarily mean that they had actually made the invention, but that they had a complete idea of the invention and provided a description of it that could be understood by one of skill in the art.
5. I believe that I am qualified by my education and training to attest to what one skilled in the art would have understood from reading the application as of September 10, 1990. In 1990, I was well experienced in the molecular biology of type I transmembrane receptors. Type I transmembrane receptors have an amino terminal extracellular region followed by a transmembrane domain and then an intracellular or cytoplasmic domain. Thus, it is the amino, rather than the carboxy, end of a type I transmembrane receptor that is exposed on the surface of a cell. For example, we had recently cloned and expressed the c-kit tyrosine kinase receptor protein, a type I transmembrane receptor (Williams et al., Cell, 63:167-174, 1990). In addition, I had worked on elucidating the relationship between the IL-4 receptor (also a type I transmembrane receptor) and other cytokine receptors. (Cosman et al., Trends in Biol. Sci. 15:265-270, 1990) The extracellular, ligand-binding domain of the IL-4 receptor showed homology to several other cytokine receptors. This homology allowed us to define a new class

of cytokine receptors. Starting in 1988, I also initiated a project at Immunex that resulted in the cloning of a number of cell surface receptors and their ligands. During the course of this work, I had occasion to make DNA constructs encoding soluble forms of type I transmembrane receptors as well as insoluble chimeric receptors containing the extracellular domain of one receptor fused to the transmembrane and cytoplasmic domains of a different receptor.

6. I note the following points as background to the discussion below. I read the application as being concerned with two tumor necrosis factor binding proteins ("TNF-BP"), one about 55kd in size and one about 75kd in size. The application's discussion of these two TNF binding proteins is consistent with what was known in the art as of September 10, 1990, i.e. that there were two membrane bound TNF receptors (TNFR) of approximately these sizes. The former is also variously referenced in the literature as TNFR I, 55 kd TNFR, or p55 TNFR. The latter is also variously referenced in the literature as TNFR II, 75 kd TNFR, or p80 TNFR.
7. Both of these receptors are type I transmembrane receptors. This is confirmed by the description in the application, which describes both of these TNF receptors as membrane-bound, meaning that they are anchored in the cell membrane. Each receptor contains an extracellular region, a transmembrane region, and an intracellular region. Figure 1 of the application displays the complete nucleotide and amino acid sequence of the 55 kd TNFR and also identifies its extracellular region (amino acids -28 to 182, corresponding to nucleotides 0 to 633), *ASZ*
intracellular transmembrane region (amino acids 182-201) and ~~extracellular~~ *7/17/07* region (amino acids 202-426). Figure 4 of the application displays a partial nucleotide and

amino acid sequence corresponding to the p75 TNFR. The membrane-bound nature of the TNF binding proteins and their potential truncation into soluble fragments is described in the application at page 7, lines 13-16, which states:

In more detail, the proteins of the present invention are non-soluble proteins, i.e. for example membrane proteins or so-called receptors, and soluble or non-soluble fragments thereof, which bind TNF (TNF-BP) . . .

8. One of skill in the art as of September 10, 1990 would have understood that the application used the term “soluble fragment” to mean a fragment of the full length receptor missing the intracellular and transmembrane regions. See, for example, the explanation of “soluble” as meaning “non-membrane bound” at page 3, lines 14-16 of the application: (“Moreover, the TNF-binding proteins described in the state of the art are *soluble, i.e. non-membrane bound*, TNF-BP. . .”). Thus, the term “soluble fragment” refers to the extracellular domain of a TNF receptor or fragments of this domain. For most, if not all, type I receptors that I was aware of as of 1990, the extracellular domain is the ligand-binding portion of the protein. Thus, one of skill in the art would have expected that the extracellular region of TNFR would bind to TNF.
9. The application’s use of the term “soluble fragment” is consistent with how the term was used in the art at the time. For example, see Deen *et al. Nature* 331(6151):82-84, 1988. Deen *et al.* showed that introducing a termination codon at the boundary between the sequence encoding the extracellular domain and the transmembrane domain would result in a soluble form of the CD4 receptor protein. This extracellular domain of CD4 was secreted from transfected cells into the cell culture, and could inhibit binding of HIV to CD4+ T cells (see Figure 3 of Deen *et al.*).

10. Anyone skilled in the art at the time would also have known that the application contemplated the *extracellular region* of the TNF binding protein as *a particular, specifically described example of a soluble fragment*. This fact is clearly conveyed by the following statements from the application:

DNA sequences which code for *soluble protein fragments* are, for example, those which extend from nucleotide -185 to 633 or from nucleotide -14 to 633 of the sequence given in Figure 1. (page 10, line 19-23) [emphasis added]

In fact, "nucleotide -14 to 633 of the sequence given in Figure 1" is a sequence which encodes the entire extracellular domain of p55 TNFR, including the signal sequence (as well as some additional upstream nucleotide sequence).

A DNA fragment which contained only the cDNA coding for the *extracellular part* of the 55 kD TNF-BP (amino acids -28 to 182 according to FIG. 1) was obtained by PCR technology . . . (page 37, lines 14-18) [emphasis added]

Analogously to the procedure described in Example 9, the cDNA fragment coding for the *extracellular region* of the 55 kDa TNF-BP was amplified in a polymerase chain reaction, . . . (page 42, lines 5-8) [emphasis added]

11. One of skill in the art at the time would have understood the following statement in the application (at page 3, line 35 through page 4, line 3) to be describing fusions of a "soluble fragment" of a TNF binding protein (either the 55 kd or 75 kd TNF receptor) to the portions of the heavy chain of human immunoglobulin described below:

This invention comprises DNA sequences which combine two partial DNA sequences, one sequence encoding soluble fragments of TNF binding proteins and the other partial sequence encoding all domains except the first domain of the constant region of the heavy chain of human immunoglobulin IgG, IgA, IgM, or IgE, and the recombinant proteins encoded by these sequences.

12. One of skill in the art at the time would also have known that the application contemplated the *extracellular region* of the TNF binding protein as *a particular, specifically described example of a soluble fragment to be fused to a portion of an immunoglobulin*. This fact would have been clearly understood from reading the entirety of the application, especially Example 11, which describes amplifying the cDNA fragment coding for the extracellular region of the 55 kd TNFR and ligating this cDNA fragment to a pCD4-Hy3 vector (which is described at page 17, lines 18-31 as containing DNA encoding an immunoglobulin fragment consisting of all domains except the first domain of the constant region of the heavy chain):

. . . the cDNA fragment coding for the extracellular region of the 55 kDa TNF-BP was amplified in a polymerase chain reaction. . . This cDNA fragment was ligated in the pCD4-Hy3 vector [DSM 5523; European Patent Application No. 90107393.2; Japanese Patent Application No. 108967/90; U.S. patent application Ser. No. 510773/90] from which the CD4-cDNA had been removed via the SstI restriction cleavage sites. (page 42, lines 6-25)] [emphasis added]

13. I disagree with the Office Action's statements at pages 8 and 9 that:

While the sequence of the entire extracellular domain of the 75kD TNF receptor was publicly available in the references of Smith (1990) and Dembic (1990) at the time of filing of the instant application, there is no description in the instant specification of these specific full-length sequences, or any description that suggests using these full-length sequences in the claimed fusion proteins. [pages 8-9 of Office Action]

While I would agree that the full length amino acid sequence disclosed in Smith (1990) or Dembic (1990) is not reproduced amino acid by amino acid, there is a description in the application of using fragments of such full length sequences in fusion proteins.

On the basis of the thus-determined sequences *and of the already known sequences for certain receptors*, those partial sequences which code for soluble TNF-BP fragments can be determined and cut out from the complete sequence using known methods [42].
[emphasis added]

Specification, page 14, lines 32-36. This language, coupled with the citation of the Smith reference within the specification, shows that the inventors were aware of the published sequence of p75 TNFR and intended to use TNF-binding fragments of such known sequences in practicing their invention.

14. I strongly disagree with the following statement at page 10 of the Office Action:

Applicants' disclosure at the time of filing would not have lead the skilled artisan to the particular species of fusion protein comprising the entire extracellular domain found in Smith.
[page 10 of Office Action]

Using the entire extracellular domain of a known and referenced type I transmembrane receptor protein is exactly what the disclosure would lead the skilled artisan to do.

15. There is a description in the application of the specific full length sequence of the 75 kD TNFR. The citation to the Smith (1990) article at page 10, lines 9-10 of the application indicates that the Applicants knew of the Smith (1990) article when they drafted the application.

16. As of September 10, 1990 one of skill in the art would look to publications, such as the Smith (1990) article, to complete the sequences of partial cDNAs. In fact, I cannot imagine anyone of skill in the art ignoring publicly available sequence, and refusing to take advantage of it to complete a partial cDNA sequence. It is disclosed in the application (page 35, lines 22-33) that Figure 4 is a partial cDNA sequence. Since the amino acid sequence of Figure 4 is almost identical (almost 99% identical) to that of Smith, it would be clear to one of skill in the art that the

protein represented by the Figure 4 sequence was the same protein described in Smith. Attached as Exhibit D is an alignment of the Figure 4 sequence with the complete sequence of p75 TNFR to illustrate this point. Further, the disclosure (page 33, lines 7-19) of the following 18-mer as the amino terminal peptide of p75 was also consistent with this conclusion:

Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys.

17. For the reasons stated above in paragraphs 6-16, there is a description in the application of using the entire extracellular region of a TNFR, including the 75 kd TNFR, in the claimed fusion proteins. Although the working examples exemplify a fusion protein comprising the entire extracellular region of the 55 kd TNFR, it is readily apparent that the application's description applies equally to the 75 kd TNFR. As the application states:

Since the invention has been described hereinbefore in general terms, the following Examples are intended to illustrate details of the invention, but they are not intended to limit its scope in any manner.

Specification, page 20, lines 27-30. Put another way, it would be unreasonable to conclude that this description of soluble fragments of TNF binding proteins applied only to the 55 kd TNFR and not the 75 kd TNFR. Thus, the application clearly contemplated that one example of a fusion protein contained the entire extracellular region of the 75 kd TNFR and a portion of an immunoglobulin.

18. Upon reading the application as of September 10, 1990, one of skill in the art would not have arrived at the same factual conclusion that the Office Action reached at page 9 regarding the reference to Smith.

The phrase "such a deletion" must refer to the "deletions" recited in the previous sentence, which are deletions made to the nucleotide sequence of Figure 1 or Figure 4. Therefore, this paragraph refers solely to nucleotide

sequences with deletions of one or more nucleotides to the sequences given in Figure 1 or Figure 4, and the proteins encoded by said nucleotides.

The stated interpretation of the reference to Smith, i.e. that Smith must disclose a fragment of the amino acid sequence of Figure 4, is illogical for the following reasons.

19. First, the application informs the reader at page 10, lines 23-26 that the sequence of Figure 4 is a partial sequence, not a complete sequence (as the Office Action points out, Figure 4 is missing a portion of the N-terminal sequence). There is no soluble fragment sequence disclosed in Smith (1990) which would be a fragment of Figure 4. Thus, the reference to Smith (1990) cannot be referring solely to a sequence that is a deletion of Figure 4 because, if anything, Smith (1990) is more complete. Clearly, then, Smith (1990) must have been referenced for another reason.
20. Second, the Office Action takes the sentences citing Smith (1990) out of the context of the entire paragraph. When the entirety of the paragraph is read, one sees that the paragraph begins with a statement indicating that this paragraph is describing soluble and non-soluble fragments of TNF binding proteins. Thus, one of skill in the art would have concluded that the citation to Smith (1990) was a reference to whatever soluble or non-soluble fragments of TNF binding proteins were described in the article. The relevant paragraph is reproduced in its entirety below:

In addition thereto, the present invention is also concerned with DNA sequences coding for proteins and soluble or non-soluble fragments thereof, which bind TNF. Thereunder there are to be understood, for example, DNA sequences coding for non-soluble proteins or soluble as well as non-soluble fragments thereof, which bind TNF, such DNA sequences being selected from the following

- (a) DNA sequences as given FIG. 1 or FIG. 4 as well as their complementary strands, or those which include these sequences;
- (b) DNA sequences which hybridize with sequences defined under (a) or fragments thereof ;
- (c) DNA sequences which, because of the degeneracy of the genetic code, do not hybridize with sequences as defined under (a) and (b), but which code for polypeptides having exactly the same amino acid sequence.

That is to say, the present invention embraces not only allelic variants, but also those DNA sequences which result from deletions, substitutions and additions from one or more nucleotides of the sequences given in FIG. 1 or FIG. 4, whereby in the case of the proteins coded thereby there come into consideration, just as before, TNF-BP. One sequence which results from such a deletion is described, for example, in Science 248, 1019-1023, (1990).

Specification, page 9, line 19 through page 10, line 10. Despite differences between the sequences disclosed in the application and those in the Smith (1990) article, I would interpret this paragraph to mean that the Smith sequence was contemplated by the inventors because the Smith (1990) article is specifically cited.

21. Third, one skilled in the art would have read the entire application, not just the quoted paragraph, in light of the disclosure of the cited Smith (1990) article. Thus, such a skilled person would have read all of the application's description relating to soluble and insoluble fragments of TNF binding proteins in light of the Smith (1990) article's disclosure of the entire extracellular region, the transmembrane region, and the intracellular region of the 75 kd TNFR, as well as its disclosure of an N-terminal cysteine-rich region of amino acids 1-162 that is a fragment of the extracellular region (see page 1020; lower right column to

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page 1021; upper left column and Figure 3 at page 1021). In 1990, it was common practice to use what is published to aid in assembling a full length sequence.

22. For these reasons above, I disagree with the statement at page 9 of the Office action that:

Therefore, the reference to Smith in the specification does not refer to a nucleotide sequence encoding the full-length extracellular domain of the receptor disclosed in Smith.

23. Moreover, one of skill in the art would not have read the application by itself without reference to any other known information. Instead, such a person would have read the application in view of what was known in the art at the effective filing date. One of skill in the art would have noted that Dembic *et al.*, *Cytokine* 2(4):231-7, 1990, published by some of the same authors as the inventors on the application, disclosed the entire sequence of the mature 75 kd TNFR, which was the same sequence as in Smith (1990), and the same extracellular region (page 232 to page 233, upper left column; and Figure 1 at page 232). The skilled person would have looked to this publication for its disclosure of the entire extracellular region of the 75 kd TNFR, for the reasons discussed above that the application references such extracellular region as a specific type of contemplated soluble fragment. Thus, one of skill in the art would have had no doubt that the inventors were in possession of the entire p75 sequence as of September 10, 1990.
24. Thus, for all of the reasons discussed above, one skilled in the art at the time would have understood that the application contemplated that the entire extracellular region of p75 TNFR was a specific example of a soluble fragment of a TNF binding protein. This skilled person, in reading the portions of the application addressing fusion proteins containing a soluble fragment of TNF

binding protein, would have looked to the publications at the time, including the Smith (1990) article cited in the application and the Dembic (1990) article published by the inventors, for their disclosure of the sequence of the extracellular region of p75 TNFR. One of skill in the art would moreover have looked to the publications at the time for whatever they disclosed with respect to soluble and insoluble fragments of p75 TNFR. If the sequence is available, of course one of skill at the time would have used such a sequence to construct a clone. This is especially the case here, where it is indicated in the specification that the Smith (1990) article is a source of information about soluble fragments of the TNF-BP that can be used, and it would be a straightforward matter to apply what is already available in the art.

25. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. § 1001), and may jeopardize the validity of the application or any patent issuing thereon.

Dated: May 22, 2007

Stewart David Lyman
Stewart David Lyman

EXHIBIT A**CURRICULUM VITAE**

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EDUCATION:

<u>Degree</u>	<u>Institution</u>	<u>Discipline</u>
B.S. (Magna cum laude)	State University of New York at Albany, Albany, NY	Biology
Ph.D.	McArdle Laboratory for Cancer Research University of Wisconsin Madison, WI	Oncology
Dissertation:	"Genetic and Metabolic Factors that May Affect the Biological Activity of Xenobiotics in Mice"	

EMPLOYMENT HISTORY:

2004 – present: Manager, Lyman BioPharma Consulting LLC

2002: Amgen Inc.

1988-2002: Immunex Corporation:

1999-2002: Director, Extramural Research

1993-1999: Senior Staff Scientist

1988-1992: Staff Scientist

Project Chair, creator of the Receptors in Search of Ligands (RISOL) Project, 1989-1994. Receptors used as tools to clone novel ligands.

Project Chair of the Signal Transduction Project, 1990-1991.

Project Chair of the Flt3 Ligand Development Project, 1994-1996.

Project Co-Chair of the Expressed Sequence Tag Project, 1995-1996.

Project Co-Chair of the Core Pipeline Technology Project, 1997-1999.

Project Co-Chair of the Basic Biology Project, 1999.

1984-1988: Post-doctoral Fellow: Fred Hutchinson Cancer Research Center, Seattle, WA. Laboratory of Dr. Larry Rohrschneider. Studied the mechanism of cellular transformation by the *v-fms* oncogene.

PROFESSIONAL ASSOCIATIONS:

American Association for the Advancement of Science

RESEARCH INTERESTS:

Identification of novel growth factors; role of growth factors in development of the hematopoietic and immune systems, role of growth factors in oncology.

HONORS AND AWARDS:

Regents Scholarship, SUNY Albany, 1973-1977
Biological Honor Society, SUNY Albany, 1976-1977
Danforth Fellowship nominee, SUNY Albany, 1977
U.S. Public Health Service National Research Service Award
McArdle Laboratory for Cancer Research, 1977-1982
NIH Postdoctoral Training Grant in Viral Oncology
University of Washington, January-September 1985
Individual National Research Service Award,
National Cancer Institute, October 1985-December, 1987.
Visiting Scientist Fellowship, Molecular Biology Computer Research
Resource, Dana-Farber Cancer Institute, Boston, MA; December 1986

ADVISORY BOARDS:

Advisory Board Member "Modern Drug Discovery", 1998 - 2004.

AWARDED PATENTS AND PATENT APPLICATION FILINGS:

U.S. Patent #5,512,457 – Cytokine designated Elk ligand
U.S. Patent #5,627,267 – Cytokine designated Elk ligand
U.S. Patent #5,670,625 – Elk ligand fusion proteins
U.S. Patent #5,728,813 – Antibodies directed against Elk ligand
U.S. Patent #5,554,512 – Ligands for Flt3 receptors
U.S. Patent #5,843,423 – Flt3 ligand stimulation of hematopoietic cells
U.S. Patent #6,190,655 – Flt3 ligand uses for exogenous gene transfer
U.S. Patent #6,540,992 – Methods for using elk-L to enhance neuronal survival
U.S. Patent #6,555,520 – Human TSLP DNA and polypeptides
U.S. Patent #6,630,143 – Antibodies against flt3 ligand
U.S. Patent #6,632,424 – Human flt3 ligand
U.S. Patent #6,762,030 – Ligand for CD7, and methods for use thereof
U.S. Patent #6,919,206 – Medium containing flt3 ligand for culturing hematopoietic cells
U.S. Patent #6,994,989 – FLK-1 binding proteins
U.S. Patent #7,041,282 – Ligands for flt3 receptors
U.S. Patent #7,045,128 – Antibodies against flt3 ligand

WO 92/00376 – The *c-kit* ligand (Steel factor)
WO 97/17442 – Novel VEGF related ligand for flk-1/KDR receptor
WO 99/33984 – V197 Polypeptide

WO 99/33983 – V201 Polypeptide
WO 99/33877 – V196 Polypeptide

INVITED TALKS AT MEETINGS:

Biology of IL-4 Receptor:

FASEB conference on Receptors, June 1990

Biotechnological Applications in the 1990's, UC-Irvine, Irvine, CA, May 1990

Biology of Steel Factor (c-kit Ligand):

Armand Hammer Workshop: Regulation of Hematopoietic Stem Cells, La Jolla, CA, October 1990

Nargis Dutt Memorial Symposium: Cytokines in Clinical Medicine, UC-Irvine, Irvine CA October 1990

Stromal Regulation of Hematopoiesis, Bethesda, MD, June 1991

Blood Cell Growth Factors Meeting, Beijing, China, August 1991

American Society for Pediatric Hematology/Oncology, Chicago IL, September 1991

AACR 43rd Annual Symposium on Fundamental Cancer Research: "Growth Factors and their Receptors in Cancer: Basic Mechanisms and Therapy" Houston, Texas, November 1991

Biology of Flt3 Ligand:

Plenary Session, 1993 ASH meeting, St. Louis, MO. December, 1993

Keystone Hematopoiesis Conference, Breckenridge, CO January, 1994

Advances in Bone Marrow Transplantation, Valhalla, NY March 7, 1994

Advances in Hematopoiesis Conference, Tokyo, Japan July 1994

The Metcalf Forum: Polyfunctionality of Hematopoietic Regulators, Dublin, Ireland September 1994

Mehdi Tavissoli Memorial Symposium: Hematopoietic Stem Cells, Reno, Nevada, November 1994

Taniguchi Foundation Symposium: Regulation of Hematopoietic Stem Cells, Osaka, Japan, December 1994

9th Symposium, Molecular Biology of Hematopoiesis, Genoa, Italy, June 24-27, 1995

International Society for Experimental Hematology, Dusseldorf, Germany, August 26-31, 1995

International Symposium on Bone Marrow Transplantation: Basic and Clinical Studies, Tokyo, Japan, October 9-10, 1995

American Association for Cancer Research Satellite Symposium: Cytokines and Cytokine Receptors, Lake George, NY, October 1995

Southern Blood Club, New Orleans, LA, February 1, 1996

Wilsede Conference, Human Leukemia Meeting, Hamburg, Germany, June 14-18, 1996

Research Trends in Hematopoietic Cell Culture, Tokyo, Japan, August 26-28, 1996

Biological Therapy of Cancer, Munich, Germany, June 11-14, 1997

IBC conference "Hematopoietic Stem Cells" San Diego, CA, June 23-24, 1997

International Society for Experimental Hematology, Cannes, France, August 24-28, 1997

Mini-symposium: Tyrosine kinase receptors, University of Lund, Sweden, August 29, 1997

PUBLICATIONS (Abstracts not included):

1. **Lyman, S.D.**, Poland, A., and Taylor, B.A. Genetic polymorphism of microsomal epoxide hydrolase activity in the mouse. J. Biol. Chem. 255, 8650-8654, 1980.

2. **Lyman, S.D.**, and Poland, A. Effect of the brachymorphic trait in mice on xenobiotic sulfate ester formation. *Biochem. Pharm.* 32, 3345-3350, 1983.
3. Jordan, V.C., Bain, R.R., **Lyman, S.D.**, and Brown, R.R. Analysis of Tamoxifen and its metabolites. In: Drug Determination in Therapeutic and Forensic Context, Ed. by E. Reid and I.D. Wilson, Plenum Press, p. 219-225, 1984.
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6. **Lyman, S.D.**, and Jordan, V.C. Possible mechanisms for the agonist actions of Tamoxifen and the antagonist actions of MER-25 (Ethamoxxytriphethyl) in the mouse uterus. *Biochem. Pharmacol.* 34, 2795-2806, 1985.
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11. **Lyman, S.D.** and Rohrschneider, L.R. The kinase activity of the *v-fms* encoded protein has a low pH optimum. *Oncogene Research* 4, 149-155, 1989.
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with a unique subset of cytoplasmic signaling proteins is induced by the Steel factor. *Mol. Cell. Biol.* 11, 3043-3051, 1991.

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EXHIBIT B
PENDING CLAIMS

Claims 1-61 (canceled)

62. (previously presented) A protein comprising

(a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human TNF, (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel, and (iii) comprises the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10); and

(b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region;
wherein said protein specifically binds human TNF.

Claims 63-101 (canceled)

102. (previously presented) The protein of claim 62, wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8).

103. (previously presented) The protein of claim 102, wherein the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

Claim 104 (canceled)

105. (previously presented) The protein of claim 62, wherein said human immunoglobulin IgG heavy chain is IgG₁.

106. (previously presented) A protein comprising

(a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human TNF, (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-

polyacrylamide gel, and (iii) comprises the amino acid sequences

LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10), LCAP (SEQ ID NO: 12), VFCT (SEQ ID NO: 8), NQPQAPGVEASGAGEA (SEQ ID NO: 9) and VPHLPAD (SEQ ID NO: 13),

wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8); and

(b) all of the domains of the constant region of a human IgG₁ heavy chain other than the first domain of the constant region;

wherein said protein specifically binds human TNF.

107. (previously presented) A recombinant protein encoded by a polynucleotide which comprises two nucleic acid subsequences,

(a) one of said subsequences encoding a human TNF-binding soluble fragment of an insoluble human TNF receptor protein having an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel, said soluble fragment comprising a the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10), and

(b) the other of said subsequences encoding all of the domains of the constant region of the heavy chain of a human IgG immunoglobulin other than the first domain of said constant region,

wherein said recombinant protein specifically binds human TNF.

Claims 108 and 109 (canceled)

110. (previously presented) The protein of claim 107, wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8).

111. (previously presented) The protein of claim 110, wherein the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

Claim 112 (canceled)

113. (previously presented) The protein of any one of claims 107, 110 or 111, wherein said human immunoglobulin heavy chain is IgG₁.

114. (previously presented) A pharmaceutical composition comprising the recombinant protein of any of claims 62, 107, 134 or 135 and a pharmaceutically acceptable carrier material.

Claims 115-118 (canceled)

119. (previously presented) The protein of claim 62, wherein the protein is purified.

120. (previously presented) The protein of claim 62, wherein the protein is produced by CHO cells.

121. (previously presented) The protein of claim 62, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human immunoglobulin IgG₁ heavy chain other than the first domain of the constant region.

Claim 122 (canceled)

123. (previously presented) The protein of claim 62, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4Hy1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSM 5314) or by pCD4-Hy3 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSM 5523).

124. (previously presented) The protein of claim 105, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4Hy1 vector (deposited at Deutschen

Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5314).

125. (previously presented) The protein of claim 106, wherein the protein is purified.

126. (previously presented) The protein of claim 106, wherein the protein is produced by CHO cells.

127. (previously presented) The protein of claim 106, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human IgG₁ heavy chain other than the first domain of the constant region.

128. (previously presented) The protein of claim 106, wherein the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

129. (previously presented) The recombinant protein of claim 107, wherein the recombinant protein is purified.

130. (previously presented) The recombinant protein of claim 107, wherein the recombinant protein is produced by CHO cells.

131. (previously presented) The recombinant protein of claim 107, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human IgG₁ heavy chain other than the first domain of the constant region.

132. (previously presented) The protein of claim 107, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4Hy1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5314) or by pCD4-Hy3 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5523).

133. (previously presented) The protein of claim 113, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by the DNA insert of pCD4Hy1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, FRG under No. DSMZ 5314).

134. (previously presented) A protein consisting of

(a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human TNF, and (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel and (iii) comprises the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10),

wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO:12) and VFCT (SEQ ID NO:8), and

(b) all of the domains of the constant region of a human IgG₁ heavy chain other than the first domain of the constant region,
wherein the protein specifically binds human TNF, and
wherein the protein is produced by CHO cells.

135. (previously presented) The protein of claim 134, wherein the soluble fragment comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

136. (previously presented) The protein of claim 134, wherein the protein is purified.

137. (previously presented) A pharmaceutical composition comprising the recombinant protein of claim 105 and a pharmaceutically acceptable carrier material.

Claim 138 (canceled)

139. (previously presented) A method of binding human TNF *in vivo* comprising the step of administering to a subject the pharmaceutical composition of claim 137.

140. (currently amended) A protein comprising

(a) a human tumor necrosis factor (TNF) binding soluble fragment of the amino acid sequence encoded by the cDNA insert of the plasmid deposited with the ATCC on October 17, 2006 under accession number PTA 7942; and

(b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region;

wherein said protein specifically binds human TNF.

141. (previously presented) The protein of claim 140 consisting of the soluble fragment and all the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of said constant region.

142. (previously presented) The protein of claim 140

wherein the protein is expressed by a mammalian host cell.

143. (previously presented) The protein of claim 142, wherein the mammalian host cell is a CHO cell.

144. (previously presented) The protein of claim 142 consisting of the soluble fragment and all the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of said constant region.

EXHIBIT C

At pages 8-9, the Office Action states:

The specification does not provide evidence that Applicants were in possession of any TNF-binding soluble fragments of an insoluble 75kD TNF-binding receptor comprising SEQ ID NO: 10. While the sequence of the entire extracellular domain of the 75kD TNF receptor was publicly available in the references of Smith (1990) and Dembic (1990) at the time of filing of the instant application, there is no description in the instant specification of these specific full-length sequences, or any description that suggests using these full-length sequences in the claimed fusion proteins. While the specification cites Smith (1990) on page 10, the specification does not contemplate use of the sequence of the full-length extracellular domain of the receptor taught in Smith.

At page 9, the Office Action states:

The only paragraph in the specification that refers to Smith (1990) states:

‘That is to say, the present invention, embraces not only allelic variants, but also those DNA sequences which result from deletions, substitutions and additions from one or more nucleotides of the sequences given in Figure 1 or Figure 4, whereby in the case of the proteins coded thereby there come into consideration, just as before, TNF-BP. One sequence which results from such a deletion is described, for example in Science 248, 1019-1023, (1990)’ (pg 10, lines 1-10)

The last sentence of this paragraph is the only sentence that refers to Smith, and this sentence only refers to ‘one sequence’ in Smith that results from a ‘such a deletion’. The phrase ‘such a deletion’ must refer to the ‘deletions’ recited in the previous sentence, which are deletions made to the nucleotide sequence of Figure 1 or Figure 4. Therefore, this paragraph refers solely to nucleotide sequences with deletions of one or more nucleotides to the sequences given in Figure 1 or Figure 4, and the proteins encoded by said nucleotides. Figure 4 shows a partial cDNA sequence of the 75 kD TNF receptor having less than the full-extracellular domain; therefore any deletions made to this sequence would not achieve a nucleotide sequence encoding the full-length extracellular domain presented in Smith. Therefore, the reference to Smith in the specification does not refer to a nucleotide sequence encoding the full-length extracellular domain of the receptor disclosed in Smith.

At page 10, the Office Action states:

Applicants' disclosure at the time of filing would not have lead the skilled artisan to the particular species of fusion protein comprising the entire extracellular domain found in Smith. In absence of a description of the full-length extracellular domain of the 75 kD receptor for use in Applicants' claimed invention, Applicants did not have possession of the claimed invention at the time of filing.

At page 10, the Office Action states:

The instant specification as filed contains no reference to the teachings of Dembic, and therefore there is no teaching in the instant specification indicating that the sequences disclosed in Dembic are relevant to the proteins of the instant invention, including the claimed fusion proteins. . . . Applicants' disclosure at the time of filing would not have lead the skilled artisan to the particular species of fusion protein comprising the entire extracellular domain found in Dembic.

EXHIBIT D

GAP of: p75-P20333 check: 5724 from: 1 to: 461

p75(smith)

to: p75-EP939121 check: 2507 from: 1 to: 392

p75(EP939121)

Symbol comparison table: /apps/gcg/gcgcore/data/rundata/blosum62.cmp

CompCheck: 1102

Gap Weight:	8	Average Match:	2.778
Length Weight:	2	Average Mismatch:	-2.248
Quality:	2052	Length:	462
Ratio:	5.235	Gaps:	1
Percent Similarity:	98.977	Percent Identity:	98.977

Match display thresholds for the alignment(s):

	=	IDENTITY
:	=	2
.	=	1

p75-P20333 x p75-EP939121 April 12, 2007 13:55 ..

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51 qmccskcspgqhakvfctktsdtvcdscedstyttlwnwvpeclscgsrc 100
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1 .....SDSVCDSCEDSTYTQLWNWVPECLSCGSRG 30

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201 tstsptsrmapgavhlpqpvsrstrqhtqptpepstapstsflmpgsp 250
   |||||||||||||||||||||||||||||||||||||||
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251 aegstgdfalpvglivgtalgliliigvvnvcvmtqvkkkplclqreakv 300
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301 phlpadkargtgqgpeqqhllitapsssssslessasaldraptrnqpqa 350
   |||||||||||||||||||||||||||||||||||||||
231 PHLPADKARGTQGPQQHLLITAPSSSSSSLESSASALDRAPTRNQPPQA 280

351 pgveasgagearastgss.dsspgghgtqvnvtcivnvcsssdhssqc 399
   |||||||||||||||||||||||||||||||||||||||
281 PGVEASGAGEARASTGSSADSSPGGHGTQVNVTCIVNVCSSSDHSSQC 330

400 qasstmgtddsspsespkdqvpfskeecafrrsqletpetllgstee 449
   |||||||||||||||||||||||||||||||||||||||
331 QASSTMGTDDSSPSESPKDEQVPFSKEECAFRSQLETPETLLGSTEEKPL 380

450 plgvpdagmkps 461
   |||||||||||
381 PLGVDPDAGMKPS 392

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Brockhaus et al.

Application No. 08/444,790

Art Unit: 1646

Filed May 19, 1995

Examiner: Z. Howard

For: HUMAN TNF RECEPTOR

**SECOND DECLARATION OF STEWART LYMAN, Ph.D.,
UNDER 37 C.F.R. § 1.132**

I, Stewart Lyman, Ph.D., declare as follows that:

1. I have been informed that the applicants of the above-identified application seek priority benefit of an earlier application, European application No. 90116707.2 filed August 31, 1990 ("August 31, 1990 Priority Application"). I have read what I have been informed is an English translation of this application, attached hereto as Appendix B.
2. I have considered the contents of the English translation of the August 31, 1990 Priority Application, the knowledge and understanding of a person skilled in the art as of August 31, 1990, and the statements I made in my prior declaration entitled "Declaration of Stewart Lyman, Ph.D. under 37 C.F.R. 1.132" regarding the above-identified application and the Office Action dated February 23, 2007.
3. The portions of the above-identified application that I referenced in my prior declaration also appear in the English translation of the August 31, 1990 Priority Application, including the citation to the Smith (1990) Science article. The table attached as Appendix A shows the page and line numbers of the corresponding cited passages in the English translation of the August 31, 1990 Priority Application.
4. All of the statements that I made in my earlier declaration with respect to what a person skilled in the art would understand from reading the text of the above-identified application as of its September 10, 1990 effective filing date apply equally to what a person skilled in the art would understand from reading the English

translation of the August 31, 1990 Priority Application as of its filing date. In other words, the written description conveyed by the above-identified application as of September 10, 1990 is the same as the written description conveyed by the English translation of the August 31, 1990 Priority Application as of its filing date.

5. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. § 1001), and may jeopardize the validity of the application or any patent issuing thereon.

July 17, 2007
Date

Stewart Lyman
Dr. Stewart Lyman

APPENDIX A

Location of passage in U.S. Appl. No. 08/444,790	Location of passage in the English translation of the August 31, 1990 Priority Application
Figure 1, pages 1 and 2 of the drawings	Figure 1, pages 21 and 22
Figure 4, pages 5 and 6 of the drawings	Figure 4, pages 25 and 26
Page 3, lines 14-16	Page 3, lines 12-14
Page 3, line 35-page 4, line 3	Page 4, lines 11-16
Page 7, lines 13-16	Page 2b, lines 1-3
Page 9, line 19-page 10, line 10	Page 3a, line 13-page 3b, line 1
Page 10, lines 9-10 (citation to Smith (1990) article)	Page 3b, line 1 (citation to Smith (1990) article)
Page 10, line 19-23	Page 3b, lines 7-9
Page 10, lines 23-26	Page 3b, lines 9-11
Page 14, lines 32-36	Page 5, line 27-page 5a, line 2
Page 17, lines 18-31	Page 6, lines 20-28
Page 20, lines 27-30	Page 7, lines 24-26
Page 33, lines 7-19	Page 13a, line 23-page 14, line 1
Page 35, lines 22-33	Page 14a, lines 20-28
Page 37, lines 14-18	Page 15a, lines 8-11
Page 42, lines 5-8	Page 17a, lines 9-11
Page 42, lines 5-25 (Example 11)	Page 17a, lines 9-21 (Example 11)
Example 11, page 42, line 1-page 43, line 8	Example 11, page 17a, line 9-page 18, line 9

Soluble Tumor Necrosis Factor (TNF) Receptors Are Effective Therapeutic Agents in Lethal Endotoxemia and Function Simultaneously as Both TNF Carriers and TNF Antagonists

Kendall M. Mohler,^{1*} Dauphine S. Torrance,* Craig A. Smith,[†] Raymond G. Goodwin,* Kay E. Stremler,[§] Victor P. Fung,^{||} Hassan Madani,[‡] and Michael B. Widmer*

Departments of Immunology, [†]Biochemistry, ^{}Molecular Biology, [§]Analytical Biochemistry, ^{||}Mammalian Cell Development, and [‡]Purification Development, Immunex Corporation, Seattle, WA 98101

ABSTRACT. Two forms (monomeric or dimeric) of the extracellular, ligand-binding portion of the human p80 cell-surface receptor for TNF were used to antagonize TNF activity in vitro and in vivo. The dimeric sTNFR:Fc molecule was a more potent inhibitor of TNF than the monomeric sTNFR (50 to 1000×), as assessed in vitro by inhibition of TNF binding or bioactivity and in vivo by protection of mice from an otherwise lethal injection of LPS. Surprisingly, the dimeric sTNFR:Fc construct demonstrated a beneficial effect even when administered 3 h after a lethal LPS injection (i.e., after serum TNF levels had peaked and receded). To study the mechanism by which the soluble TNFR functions in vivo, serum TNF levels were examined in mice given LPS in the presence or absence of soluble receptor. Administration of a mortality-reducing dose of sTNFR:Fc ablated the rise in serum TNF bioactivity that normally occurs in response to LPS. However, TNF bioactivity was revealed in these "TNF-negative" serum samples when the L929 bioassay was modified by inclusion of a mAb that blocks the binding of murine TNF to the human soluble TNFR receptor. These results indicate that the absence of direct cytolytic activity in the L929 assay was caused by neutralization of TNF, rather than to an absence of TNF in the serum. Moreover, administration of either monomeric sTNFR or low doses of dimeric sTNFR:Fc actually resulted in increased serum TNF levels compared to mice given LPS but no soluble receptor. However, these "agonistic" doses of soluble receptor did not lead to increased mortality when an LD₆₀ dose of LPS was given. Thus, dimeric sTNFR are effective inhibitors of TNF and under some circumstances function simultaneously as both TNF "carriers" and antagonists of TNF biologic activity. *Journal of Immunology*, 1993, 151: 1548.

TNF is a polypeptide hormone released by activated macrophages and T cells, which mediates a wide range of biologic functions. In addition to its potential role as a regulator of the normal immune response, TNF is also thought to play a major role in systemic toxicity associated with sepsis (1–6). TNF may also be involved in the pathogenesis of AIDS (7–9) as well as a number of autoimmune and inflammatory diseases (10–13). A mole-

cule that specifically inhibits the biologic activities of TNF may thus have considerable therapeutic utility.

Soluble, extracellular, ligand-binding portions of cytokine receptors occur naturally in body fluids and are believed to regulate the biologic activities of cytokines (14–17). The importance of these molecules as cytokine regulators is underscored by the fact that several pox viruses encode proteins with structural and functional homology to the extracellular portions of the receptors for TNF and IL-1 (18–20). Considerable controversy exists concerning the type of regulatory role naturally occurring soluble cytokine receptors might perform. Although it is likely that such molecules will function as cytokine carriers in an operational sense by altering the biodistribution of the cytokine to which they bind, it is not clear whether such an interaction would serve to agonize or antagonize the biologic

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effects of the cytokine (21, 22). However, experiments in which recombinant soluble receptors have been administered *in vivo* demonstrate their potential to inhibit immune and inflammatory responses, presumably by acting as antagonists of cytokine activity (23, 24).

There are two distinct cell-surface receptors for TNF: the 80 kDa (p80) and the 60 kDa (p60) receptors, both of which bind TNF- α and TNF- β (25, 26). Given the predominantly trimeric nature of TNF (25) and the apparent requirement for cross-linking of cell-surface TNFR for signal transduction (27), it is likely that dimeric soluble receptor constructs should possess a higher affinity for TNF (28) and therefore function as considerably more potent competitive inhibitors than monomeric sTNFR.² This prediction has been verified by the results of recent experiments demonstrating superior TNF inhibitory activity of dimeric Fc fusion constructs of p60 *in vitro* (29). Although soluble forms of both monomeric and dimeric p60 TNFR have been shown to be beneficial in animal models of sepsis, no direct comparison of the *in vivo* potency of monomeric vs dimeric receptors in sepsis has been reported. In addition, little information is available concerning the mode of action of such inhibitors *in vivo*.

Monomeric and dimeric (Fc fusion protein) forms of the p80 TNFR were constructed and compared *in vitro* and *in vivo* for effects on TNF biologic activity. The results indicate that the sTNFR:Fc, but not the sTNFR, was effective in reducing mortality associated with LPS administration, at least over the concentration range tested. In addition, the sTNFR:Fc molecule can function simultaneously as both a TNF "carrier" and an antagonist of TNF biologic activity and thus inhibit the lethal effects of LPS by acting as a biologic buffer for TNF.

Materials and Methods

Mice

BALB/c female mice 8 to 10 wk old were purchased from Charles River (Wilmington, MA) and were maintained within a specific pathogen-free environment.

Construction and production of p80 sTNFR and sTNFR:Fc

Recombinant sTNFR was expressed in a CHO cell line using the glutamine synthetase selectable and amplifiable marker. For production, cells cultured to confluence in roller bottles were washed with PBS and then cultured in serum-free medium. Purification of the sTNFR from the CHO supernatant was accomplished in a single affinity

chromatography step using a mAb, M1, specific for sTNFR.

Recombinant sTNFR:Fc was expressed in CHO cells using the dihydrofolate reductase selectable and amplifiable marker. Suspension cells were centrifuged and resuspended into serum-free medium in a controlled bioreactor. The product was collected after 7 days. The sTNFR:Fc molecule was purified using protein A affinity chromatography followed by an ion-exchange step.

Concentrations of the purified sTNFR and sTNFR:Fc were determined by amino acid analysis. Endotoxin levels were determined to be <5.6 ng endotoxin/mg sTNFR or sTNFR:Fc using the Kinetic-QCL assay (Whittaker Bioproducts, Walkersville, MD) for detection of Gram-negative bacterial endotoxin. Physical characterization included SDS-PAGE, N-terminal sequencing, and immunoreactivity analyses (K. E. Stramler and H. Madani, unpublished observations). A diagrammatic representation of p80 sTNFR and sTNFR:Fc is shown in Figure 1.

Antibodies to soluble TNFR

The generation of mAb to the human p80 sTNFR has been described previously (30). M1 mAb (rat IgG 2b) and M3 (rat IgG) mAb both bind to the human p80 sTNFR but not to mouse TNFR.

Binding inhibition assay

Human rTNF- α was expressed in yeast as a protein composed of the entire coding region of mature TNF fused to an octapeptide at the N terminus, useful in affinity purification. Purified TNF was radioiodinated as described (18) to a sp. act. of 2×10^{15} cpm/mmol, without loss of biologic activity (measured in an L929 cytotoxicity assay) or receptor-binding activity (see below).

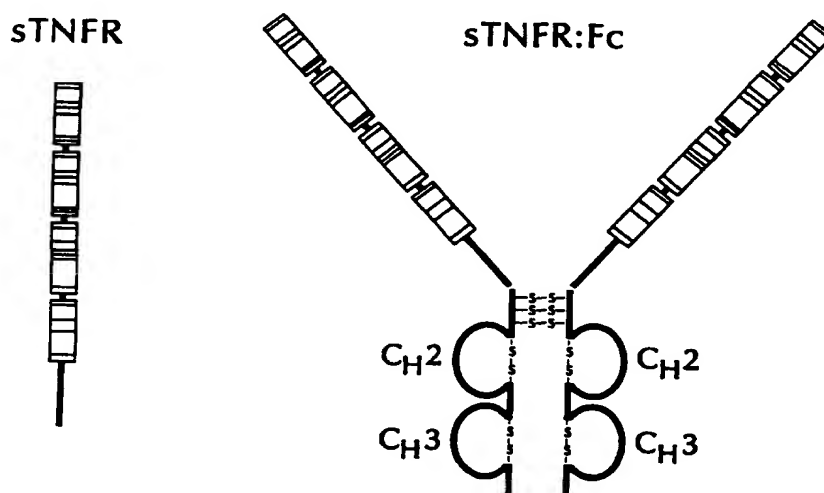
Inhibition assays were carried out as described (31). Briefly, [125 I]TNF- α (0.5 nM) was incubated in binding medium (RPMI 1640, 2.5% BSA, 50 mM HEPES buffer, pH 7.4, 0.4% Na₂S₂O₃) for 2 h at 4°C with serially diluted inhibitors (human sTNFR:Fc, sTNFR monomer, or unlabeled human rTNF- α) and 2×10^6 U937 cells. Duplicate aliquots were subsequently removed, centrifuged through a phthalate oil mixture to separate free and bound ligand, and the radioactivity was measured on a gamma counter. Nonspecific binding values were determined by inclusion of a 200× molar excess of unlabeled TNF and were subtracted from total binding data to yield specific binding values. Data were plotted and results analyzed as described (31).

L929 bioassay for TNF activity

The protocol used to measure the presence of TNF cytotoxic activity using L929 cells as targets has been described previously (32, 33). Briefly, 10 μ l of mouse serum, mouse

² Abbreviations used in this paper: sTNFR, soluble monomeric human p80 TNFR; sTNFR:Fc, recombinant fusion protein composed of soluble dimeric human p80 TNFR linked to the Fc region of human IgG1; CHO, Chinese hamster ovary.

FIGURE 1. Construction of monomeric sTNFR and dimeric sTNFR:Fc molecules. Extracellular portions of the human p80 TNFR cDNA were cloned and produced as described in *Materials and Methods*. In the dimeric sTNFR:Fc molecule three disulfide bonds are depicted. However, the disulfide bond closest to the N terminus is normally used for binding to the Ig L chain and thus, its state (i.e., free cysteine or disulfide bond) in the sTNFR:Fc fusion product is not known.



rTNF- α (Genzyme, Boston, MA), or supernatant from LPS-stimulated RAW 264.7 cells (American Type Culture Collection, Rockville, MD) was serially diluted (50%:50%, v/v) in flat bottom, 96-well microtiter plates. L929 medium (RPMI 1640 with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin) was added to each well, followed by soluble receptors, control proteins, or mAb in a total volume of 30 μ l. Ten microliters of actinomycin D was then added (final concentration of 0.1 μ g/well; Sigma, St. Louis, MO). Finally, 5×10^4 L929 cells were added to each well (final volume/well = 100 μ l) and the plates were incubated at 37°C in 5% CO₂. To prevent the influence of edge effects on the TNF bioassay, only the inner wells of each plate were utilized. All outer wells received 200 μ l of L929 medium only. After 16 h of incubation, the culture medium was removed and replaced with 200 μ l of 0.5% crystal violet in methanol/water (1/4). The plate was washed with distilled water and air dried at ambient temperature. One hundred microliters of 2% deoxycholic acid (catalog no. D-6750, Sigma) was added to each well to solubilize the crystal violet and the plates were analyzed on an ELISA plate reader at 562 nm. The negative control consisted of L929 cells in the presence of actinomycin-D. Estimates of serum TNF concentrations were obtained by comparing the TNF activity in the experimental serum samples with the activity obtained with the mouse rTNF- α standard.

LPS-induced mortality

LPS, derived from *Escherichia coli* 0127:B8 (catalog no. DF3132-25, VWR, Seattle, WA), was resuspended at 10 mg/ml in sterile saline and stored at -20°C in small aliquots. The LPS was diluted to the proper concentration and sonicated (CU-6 sonicator; Branson, Shelton, CT) for 1 min before injection. BALB/c female mice (18 to 20 g) were injected i.v. with an LD₆₀ to LD₁₀₀ dose of LPS (300 to 400

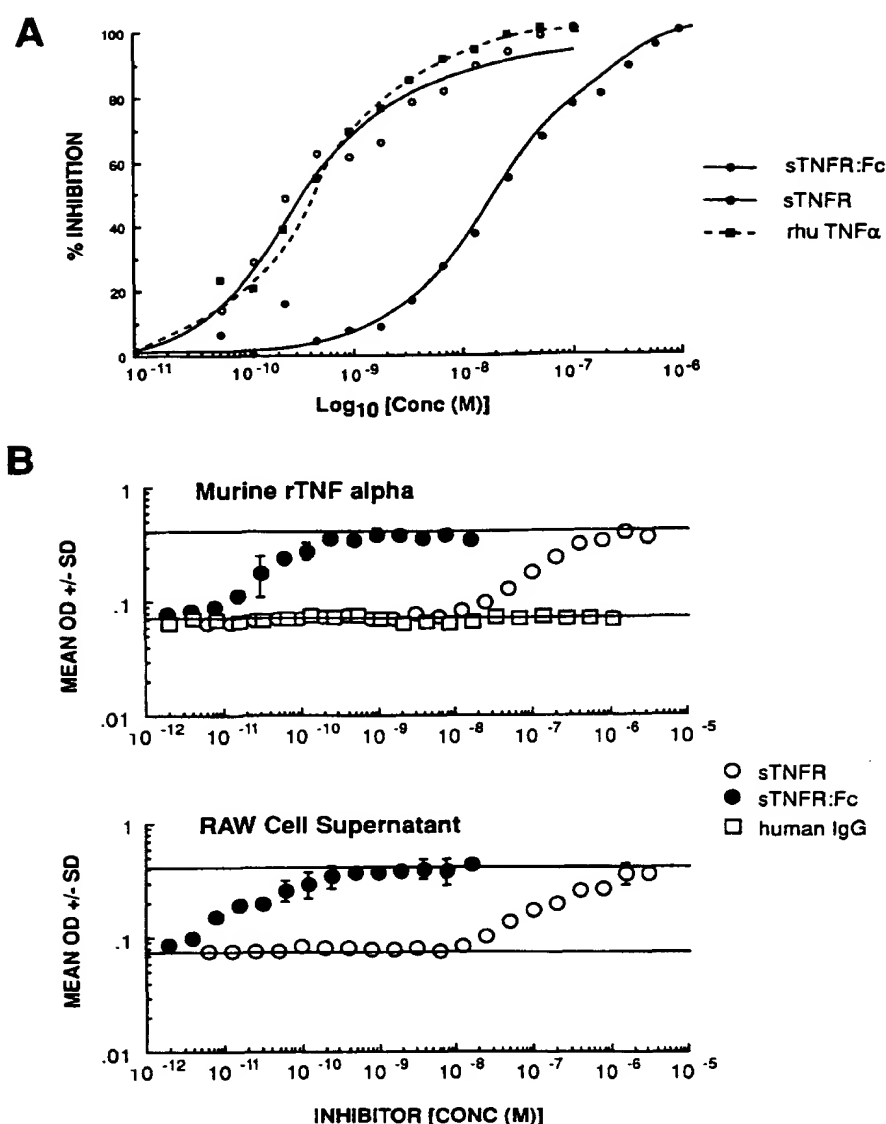
μ g) in 0.2 ml of saline. The LPS was injected either alone or in conjunction with sTNFR, sTNFR:Fc or control protein, human IgG (catalog no. I-4506, Sigma). In some experiments, mice were injected with LPS i.v. followed at 2, 3, or 4 h with an i.v. injection of soluble receptor or control protein. Survival was monitored for at least 5 days and, in some experiments, the mice were observed for a maximum of 4 wk. However, no further mortality occurred after the initial 5-day observation period.

Results

In vitro neutralization of TNF activity by soluble TNFR

The ligand binding characteristics of sTNFR monomer and sTNFR:Fc were determined by cell-based inhibition studies using ¹²⁵I-human rTNF- α and U937 cells expressing surface p80 and p60 TNFR. Results of these experiments are shown in Figure 2A. To generate a robust criterion of the relative activity of the sTNFR:Fc, we have analyzed the binding inhibition data with a simple one-site model to yield a single K_i , which reflects that concentration of inhibitor which mediates 50% inhibition of binding of TNF to cell-surface receptors. As predicted from (1) the multivalent interactions that occur between TNF ligands and receptors and (2) previous studies (29), the sTNFR:Fc ($K_i = 1 \times 10^{10}$ M⁻¹) shows ~50-fold higher affinity for the ligand than does the sTNFR monomer ($K_i = 2 \times 10^8$ M⁻¹). Thus, one might suspect that the sTNFR:Fc molecule would be a better antagonist of TNF biologic activity in comparison to the monomeric sTNFR in vitro and in vivo. To address the biologic efficacy of monomeric (sTNFR) and dimeric (sTNFR:Fc) forms of the soluble p80 TNFR, both molecules were analyzed for their ability to neutralize TNF activity in vitro in the L929 bioassay (Fig. 2B). Monomeric sTNFR and dimeric sTNFR:Fc inhibited the ac-

FIGURE 2. Comparison of TNF binding and neutralizing capability of sTNFR and sTNFR:Fc. **A**, U937 cells (2×10^6) were incubated at 4°C for 4 h with 0.5 nM 125 I-human rTNF- α in binding medium and varying concentrations of inhibitor (sTNFR:Fc, sTNFR monomer or unlabeled human rTNF- α) in a total volume of 150 μ l. Duplicate 70- μ l aliquots of the suspension were subsequently removed and microfuged through a phthalate oil mixture to separate free and bound ligand. Radioactivity was measured in a gamma counter and the data were analyzed according to a simple competitive inhibition model. **B**, a constant amount of murine rTNF- α (125 pg/ml) or natural TNF (derived from LPS-stimulated RAW cells, 1/200 dilution) was added to each well of an L929 cytotoxicity assay in the presence of varying amounts of inhibitors (sTNFR, sTNFR:Fc or human IgG). Details of the L929 cytotoxicity assay are provided in *Materials and Methods*. The OD of L929 cells in the absence of TNF is indicated by the upper solid line (mean OD approximately 0.45) and maximal lysis of L929 cells is indicated by the lower solid line (mean OD approximately 0.075).



tivity of mouse TNF (recombinant or natural) in a dose-dependent fashion; however, sTNFR:Fc was approximately 1000-fold more efficient than sTNFR. Identical results were obtained when human rTNF- α was utilized as the ligand (data not shown). Human IgG, used as a control protein, had no effect on TNF activity.

Ability of sTNFR to prevent mortality induced by LPS

We have also compared the biologic efficacy of sTNFR and sTNFR:Fc in vivo in a murine model of LPS-induced septic shock. Various doses of sTNFR:Fc or control protein (human IgG) were mixed with a lethal dose of *E. coli* LPS (400 μ g/mouse) and injected i.v. into 18- to 20-g BALB/c female mice. Survival was monitored for 5 days and the results are presented in Figure 3. Treatment of mice with LPS only or LPS and any dose of human IgG resulted in 0 to 10% long

term survival. In contrast, 90% of mice treated with LPS plus 100 μ g (1.95 nmol) of sTNFR:Fc survived. Beneficial effects of the sTNFR:Fc protein were also evident with doses as low as 10 μ g (0.2 nmol)/mouse. In similar studies we have been unable to demonstrate an effect of recombinant monomeric sTNFR on survival even when doses as high as 260 μ g (10.35 nmol) were administered (Fig. 4). However, based on the in vitro neutralizing capacity of the monomeric vs dimeric sTNFR (Fig. 2) and the dose of sTNFR:Fc required to effect survival in vivo (Fig. 3), monomeric sTNFR would be predicted to demonstrate efficacy at much higher doses (10 mg/mouse).

The ability of the sTNFR:Fc protein to provide protection when given at various times after LPS administration was also tested. Mice received a lethal dose of LPS (i.v.) followed 2, 3, or 4 h later by sTNFR:Fc (100 μ g/mouse). Two to three separate experiments were conducted for each

FIGURE 3. Administration of sTNFR:Fc prevents mortality of BALB/c mice injected with a lethal dose of LPS. Various doses of sTNFR:Fc or human IgG, as a control, were mixed with a lethal dose of LPS (400 μ g) and injected i.v. into BALB/c mice. Survival was monitored at least once a day for 5 days. In each of three separate experiments, mice treated with sTNFR:Fc at doses of 10 μ g or above demonstrated enhanced survival.

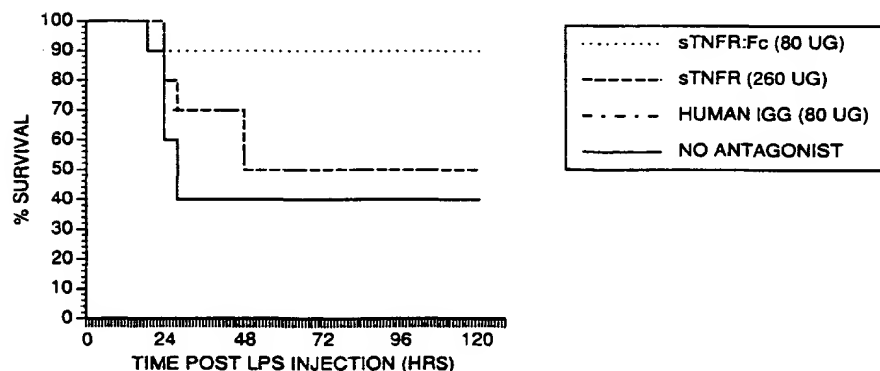
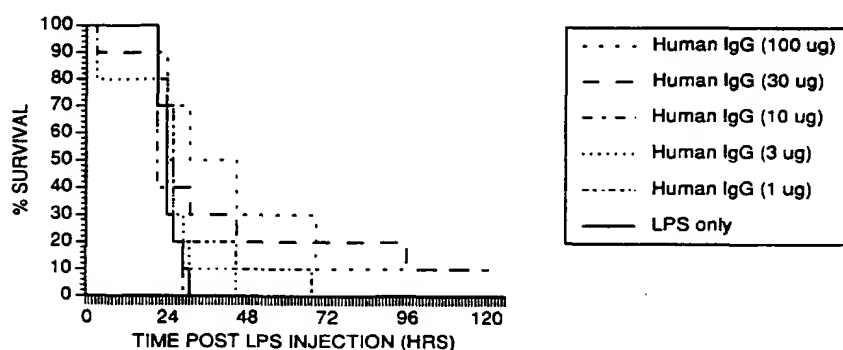
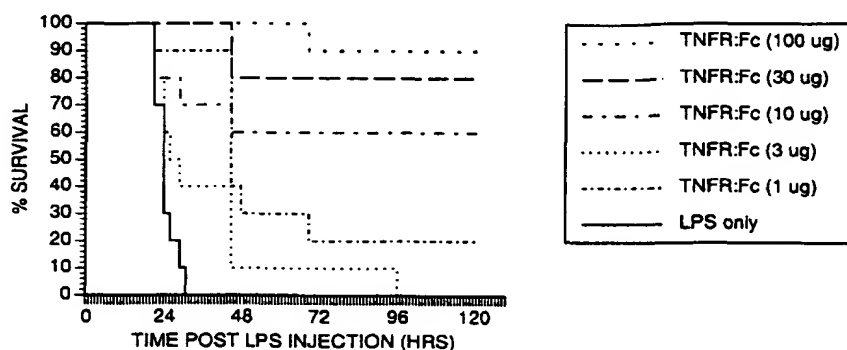


FIGURE 4. Administration of sTNFR does not affect mortality of BALB/c mice injected with a lethal dose of LPS. The procedure was identical to that described in the legend to Figure 3. *Note:* the response of mice treated with human IgG plus LPS overlaps the response of mice treated with sTNFR (260 μ g) plus LPS.

time point. All experiments provided similar results and therefore the results were pooled (Fig. 5). The results demonstrate that the administration of sTNFR:Fc was clearly beneficial even when administered up to 3 h after the injection of LPS. In the same experiment, the progression of serum TNF activity after LPS injection was determined in a subset of mice that received LPS only (Fig. 6). These experiments and previous reports (34–36) demonstrate that most of the serum TNF activity was produced during the first 2 h after LPS administration. These results demonstrate that the sTNFR:Fc protein was efficacious even when administered after serum TNF levels had peaked and receded. Thus, the efficacy of the sTNFR:Fc molecule must not be due solely to neutralization of serum TNF bioactivity.

Effect of sTNFR and sTNFR:Fc on serum TNF levels in vivo

To study the mechanism by which sTNFR:Fc protected mice from an otherwise lethal dose of LPS, the effect of the two forms of soluble TNFR on TNF activity present in the serum was examined. Mice were injected with LPS alone (400 μ g) or LPS mixed with 100 μ g of sTNFR, sTNFR:Fc, or control protein, human IgG. Serum samples were obtained 2 h after injection and assayed for TNF bioactivity (Fig. 7). Mice injected with LPS alone or LPS mixed with human IgG exhibited equivalent amounts of serum TNF activity (approximately 1 ng/ml) 2 h after LPS injection. In contrast, mice treated with LPS plus 100 μ g of sTNFR:Fc

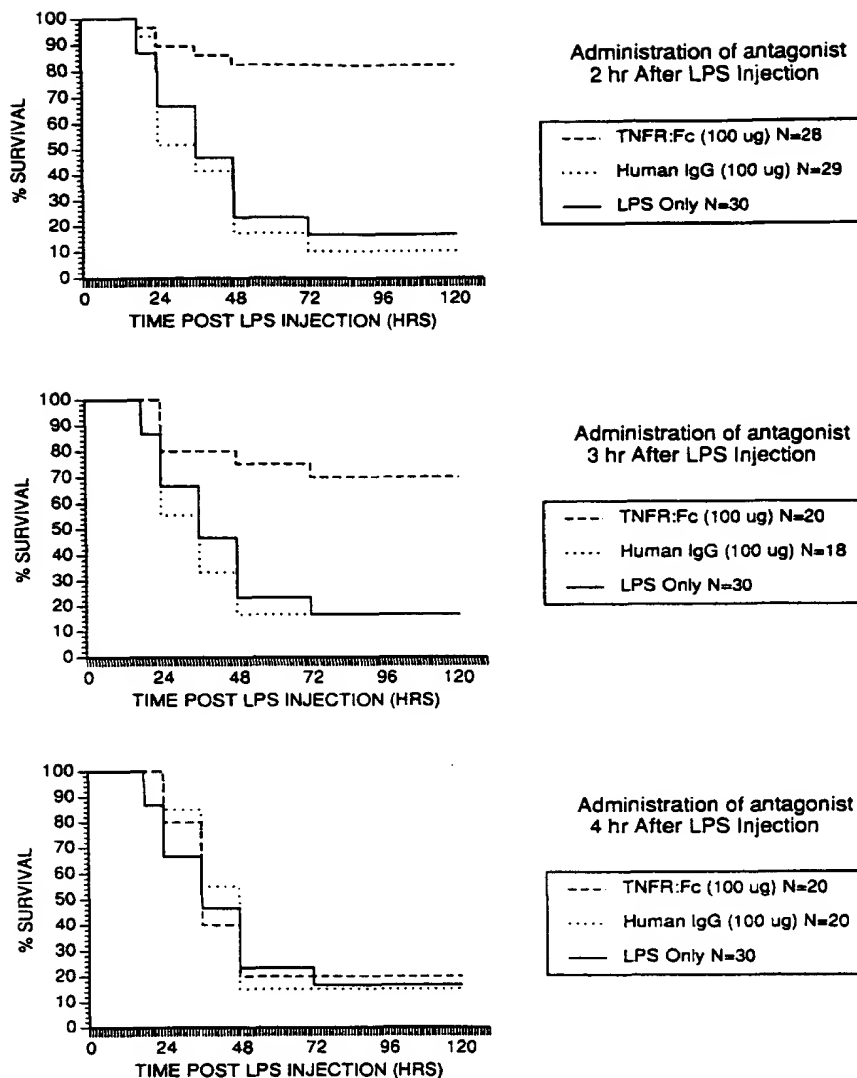


FIGURE 5. Administration of sTNFR:Fc prevents mortality of BALB/c mice even when injected 3 h after a lethal dose of LPS. At 2, 3, or 4 h after i.v. injection of a lethal dose of LPS (400 μ g), 100 μ g of sTNFR:Fc or human IgG, as a control, were injected i.v. Survival was monitored at least once a day for 5 days. The results represent a compilation of two to three separate experiments.

(which protects mice from the lethal effects of LPS injection, (Fig. 3)) had little or no serum TNF activity as assessed in the L929 assay. Somewhat surprisingly, mice treated with an equivalent dose of monomeric sTNFR (which was not efficacious in survival studies) exhibited serum TNF levels 10-fold higher (10 ng/ml) than control mice treated with LPS only or LPS plus human IgG.

Figure 8 depicts results of an experiment in which the relationship between the dose of sTNFR:Fc and serum TNF activity was examined. Sera obtained from mice injected with LPS alone or LPS plus 1 to 100 μ g of human IgG contained detectable TNF activity that titrated in a predictable fashion. Sera obtained from mice 2 h after treatment with 100 or 30 μ g of sTNFR:Fc and LPS contained little if any demonstrable TNF activity. Mice injected with 10, 3, or 1 μ g of sTNFR:Fc and LPS exhibited serum TNF activity but the sera displayed unusual characteristics. These serum samples demonstrated intermediate levels of

TNF activity, which failed to decrease even when diluted to 1/160 (Fig. 8) (data not shown). Because these results were obtained only when mice received LPS and low doses of the sTNFR:Fc, we examined the influence of the sTNFR:Fc on TNF activity in these samples.

Ability of sTNFR:Fc molecules to act as carriers of TNF

Experiments were conducted to determine the effect of blocking the TNF-binding ability of sTNFR:Fc molecules in vitro in the L929 cytotoxicity assay. To this end, we utilized a mAb (M1) that binds to the sTNFR:Fc molecule and blocks the ability of the soluble human TNFR:Fc protein to bind TNF. Another rat mAb (M3) that binds the sTNFR:Fc molecule but does not block TNF binding was used as a control. To examine the ability of M1 to block TNF binding to sTNFR:Fc proteins, constant amounts of sTNFR:Fc (200

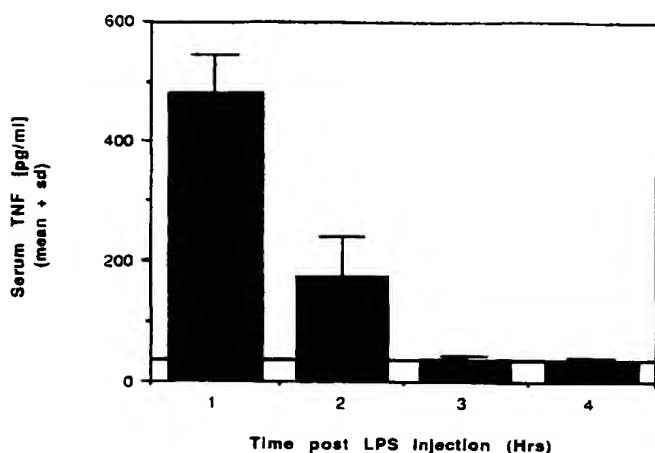


FIGURE 6. Serum TNF levels are elevated for 2 h after lethal LPS injection. Mice were injected with a lethal dose of LPS (400 μ g) and serum was obtained at 1, 2, 3, or 4 h. TNF activity was assessed by the L929 cytotoxicity assay as described in *Materials and Methods*.

ng/ml) and murine rTNF α (125 pg/ml) were added to dilutions of M1, M3, or rat IgG (Fig. 9). The ability of the sTNFR:Fc protein to neutralize the activity of TNF was reversed only in the presence of M1. In addition, full neutralization of the sTNFR:Fc protein (200 ng/ml) required a 10-fold excess (2 μ g/ml) of M1.

The effect of addition of M1 to serum obtained from mice 2 h after injection of LPS (400 μ g) mixed with 10 or 100 μ g of sTNFR:Fc was examined. As previously described (Fig. 8), the serum obtained from mice treated with 10 μ g of sTNFR:Fc demonstrated intermediate levels of activity that were not altered by dilution (Fig. 10). Addition of M1 (2 μ g/ml) to dilutions of the serum revealed the presence of additional TNF activity, which titrated in a predictable fashion. As expected, addition of control antibody (i.e., M3 or rat IgG) had no effect on the TNF activity. Furthermore, addition of M1, M3, or rat IgG had no effect on serum samples that did not contain the soluble human TNFR:Fc protein (i.e., sera obtained from mice injected with LPS and human IgG), demonstrating that the antibody did not affect the ability of mouse TNF to bind to the indicator L929 cells (Fig. 10). We have also examined serum samples from mice treated with a higher dose of sTNFR:Fc (100 μ g) and LPS. In the absence of manipulation these samples did not demonstrate TNF activity *in vitro*. However, TNF activity was revealed when serum from these mice was treated with M1 but not with M3 or rat IgG (Fig. 10). In fact, maximal activity in the L929 assay of the sera from mice injected with sTNFR:Fc (100 μ g) and LPS was still apparent at serum dilutions of 1/100, whereas sera obtained from mice treated with LPS only or LPS plus human IgG demonstrated only small amounts of TNF activity at a dilution of 1/16 (Fig. 10).

To determine whether or not sTNFR:Fc could prolong the presence of serum TNF, mice were injected with LPS and 10 or 100 μ g of sTNFR:Fc or human IgG, as described above, and serum samples were obtained at 4 h. The serum samples were assayed in the L929 bioassay in the presence and absence of M1, M3, or rat IgG (Fig. 11). As expected, sera obtained from mice injected 4 h previously with LPS alone or LPS plus human IgG did not contain serum TNF activity. However, sera obtained from mice injected with LPS plus sTNFR:Fc (10 or 100 μ g) still contained biologically active TNF, which titrated in a predictable fashion in the presence of M1 mAb. Thus, mice injected with LPS and the soluble human TNFR:Fc protein, even at therapeutic doses, retained increased levels of TNF in the serum that persisted for longer periods of time. However, depending upon the dose of sTNFR:Fc administered, the TNF activity was either (1) enhanced or (2) revealed only upon the addition of a mAb which blocked the binding of TNF to the sTNFR:Fc protein. These observations indicate that the binding of the sTNFR:Fc protein to TNF is reversible and that the inhibition of TNF activity reflects a balance between the presence of sTNFR:Fc, TNF, and endogenous TNFR (either cell surface or soluble).

The carrier function of sTNFR:Fc molecules is not detrimental to the host

As the administration of sTNFR:Fc under some circumstances produced increased levels of serum TNF (Fig. 8) that persisted for at least 4 h (Fig. 11), it was important to determine whether or not the administration of sTNFR:Fc molecules under these circumstances would lead to detrimental consequences. Mice were injected with a dose of LPS (300 μ g) which produced intermediate levels of mortality (60 to 70%), such that beneficial or deleterious effects of the TNFR could be observed. Mice treated with sTNFR:Fc at doses ranging from 10 ng to 10 μ g demonstrated equivalent or slightly better survival when compared with mice treated with LPS alone or LPS and human IgG (Fig. 12). Further experiments in which lower doses of sTNFR:Fc (100 pg to 1 μ g) were utilized yielded similar results (data not shown). Thus, administration of sTNFR:Fc in sublethal models of LPS toxicity had no detrimental consequences on the survival incidence.

Discussion

The data presented in this report demonstrate that a fusion molecule consisting of a soluble form of the extracellular portion of the p80 cell surface TNFR fused to the Fc portion of human IgG1 (sTNFR:Fc) is an effective antagonist of LPS-induced septic shock. An increased incidence of survival in mice given an otherwise lethal dose of LPS was observed when the sTNFR:Fc protein was injected 0 to 3

FIGURE 7. The effect of sTNFR vs sTNFR:Fc on serum TNF levels after co-administration of LPS in vivo. LPS (400 μ g) was mixed with 100 μ g of sTNFR, sTNFR:Fc, or human IgG and administered i.v. to BALB/c mice. Serum samples were obtained 2 h after injection and analyzed for TNF activity in the L929 cytotoxicity assay. The results were obtained from three to four separate experiments for each treatment group. The sensitivity of the TNF bioassay is approximately 50 pg/ml and is indicated by the solid line.

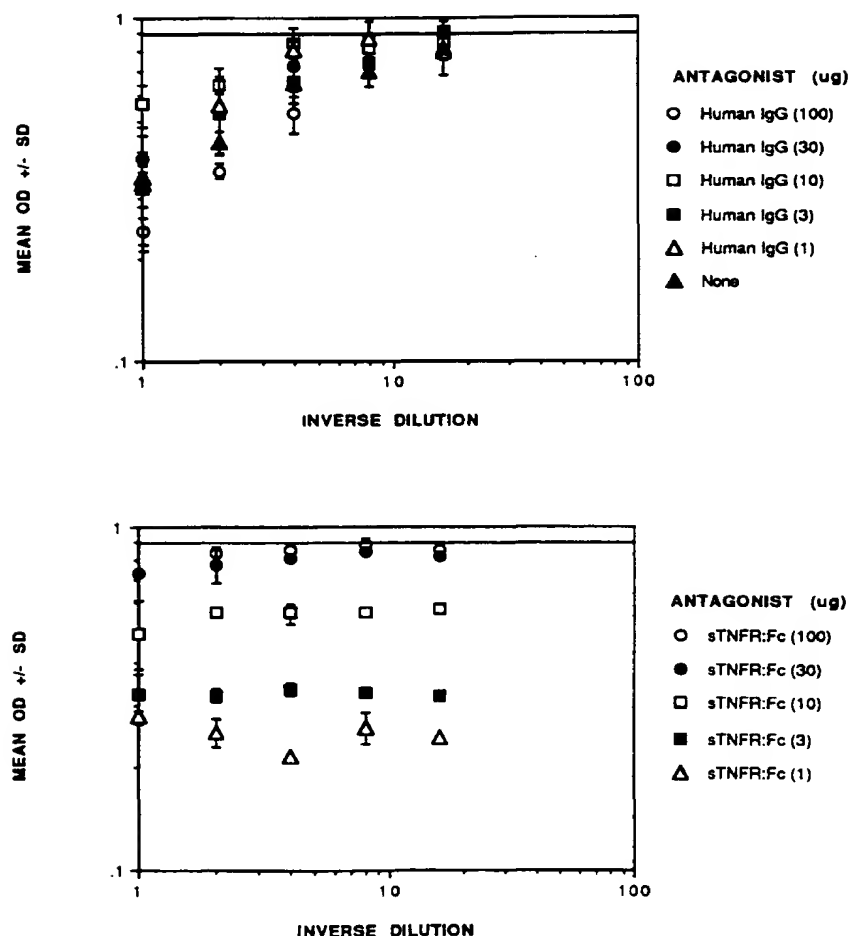
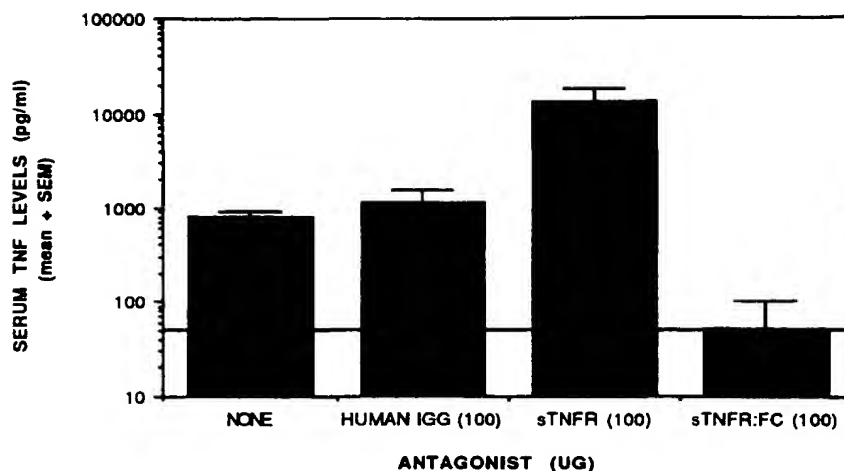


FIGURE 8. Analysis of TNF bioactivity in serum samples obtained 2 h after in vivo co-injection of LPS and sTNFR:Fc. A lethal dose of LPS (400 μ g) was mixed with varying doses of sTNFR:Fc or human IgG and injected i.v. into BALB/c mice. Serum was obtained from three mice in each group 2 h after injection. The serum for each group was pooled and analyzed for TNF activity in the L929 assay.

h after LPS administration (Figs. 3 and 5). When administered simultaneously with LPS, doses of sTNFR:Fc as low as 10 μ g (0.2 nmol)/mouse were beneficial (Fig. 3). In contrast, administration of up to 260 μ g (10.35 nmol) of the monomeric sTNFR failed to affect the incidence of mortality induced by LPS, even when the incidence of mortality in the control group was only 50% (Fig. 4). This difference

in efficacy between sTNFR:Fc and sTNFR in vivo may be explained in large part by the higher affinity of TNF for sTNFR:Fc than sTNFR, which results in a substantially greater ability of sTNFR:Fc to neutralize the biologic effects of TNF (Fig. 2). Furthermore, linkage of the sTNFR to the Fc region of Ig imparts a fivefold longer serum $t_{1/2}$ to the sTNFR:Fc molecule after i.v. injection (37), a property

FIGURE 9. Inhibition of the TNF neutralizing capacity of the human p80 sTNFR:Fc molecule by M1 but not M3 mAb. Dilutions of M1, M3, or rat IgG were added to constant amounts of sTNFR:Fc and murine rTNF- α in the L929 assay as described in *Materials and Methods*.

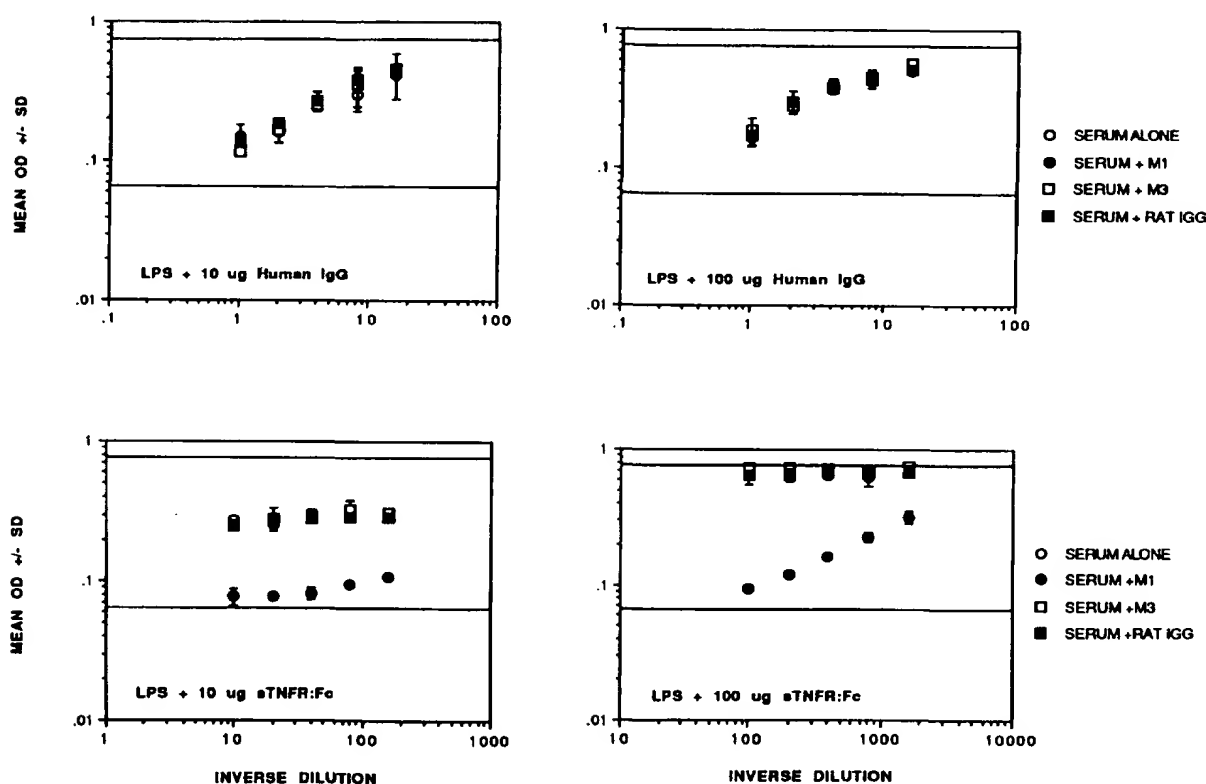
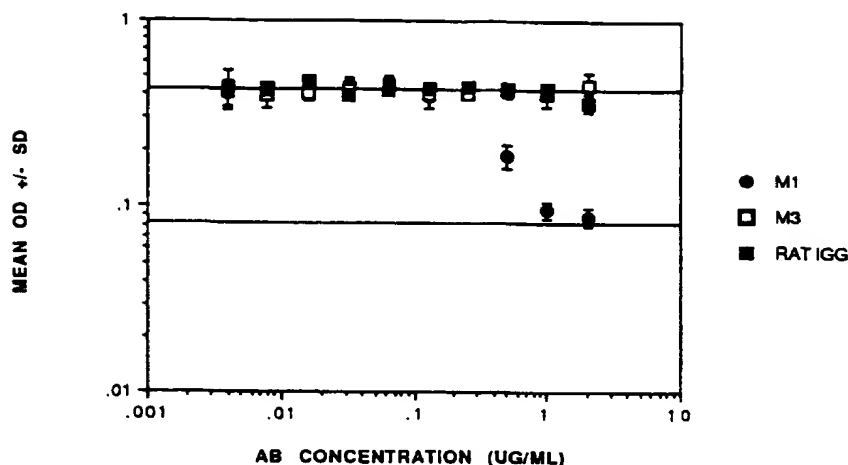


FIGURE 10. Demonstration of serum TNF activity in vitro in the L929 assay in the presence of M1 mAb. Serum was obtained from mice 2 h after injection of LPS (400 μ g) mixed with sTNFR:Fc or human IgG. The serum samples were serially diluted and assayed in the L929 cytotoxicity assay either alone or in the presence of constant amounts (2 μ g/ml) of M1, M3, or rat IgG.

that could also influence its efficacy in vivo.

The ability of sTNFR to alter the magnitude and time course of serum TNF after co-administration with LPS in vivo was examined. Sera from mice that received high, life-saving doses of sTNFR:Fc (e.g., 100 μ g) failed to exhibit significant levels of TNF bioactivity when assayed directly in the L929 cytotoxicity assay. However, further experimentation demonstrated that TNF was present in the serum but it was biologically inactive because of the con-

comitant presence of sTNFR:Fc. TNF activity in these samples was revealed in the presence of a mAb which blocked the ability of the human sTNFR:Fc molecules to bind TNF but did not interfere with the ability of TNF to bind to the murine TNFR on the surface of the L929 indicator cells (Figs. 9 to 11). These results suggest that the sTNFR:Fc protein has a relatively high exchange rate for TNF, such that once TNF is released in vitro, it can be detected if the TNF is inhibited from subsequently binding to free

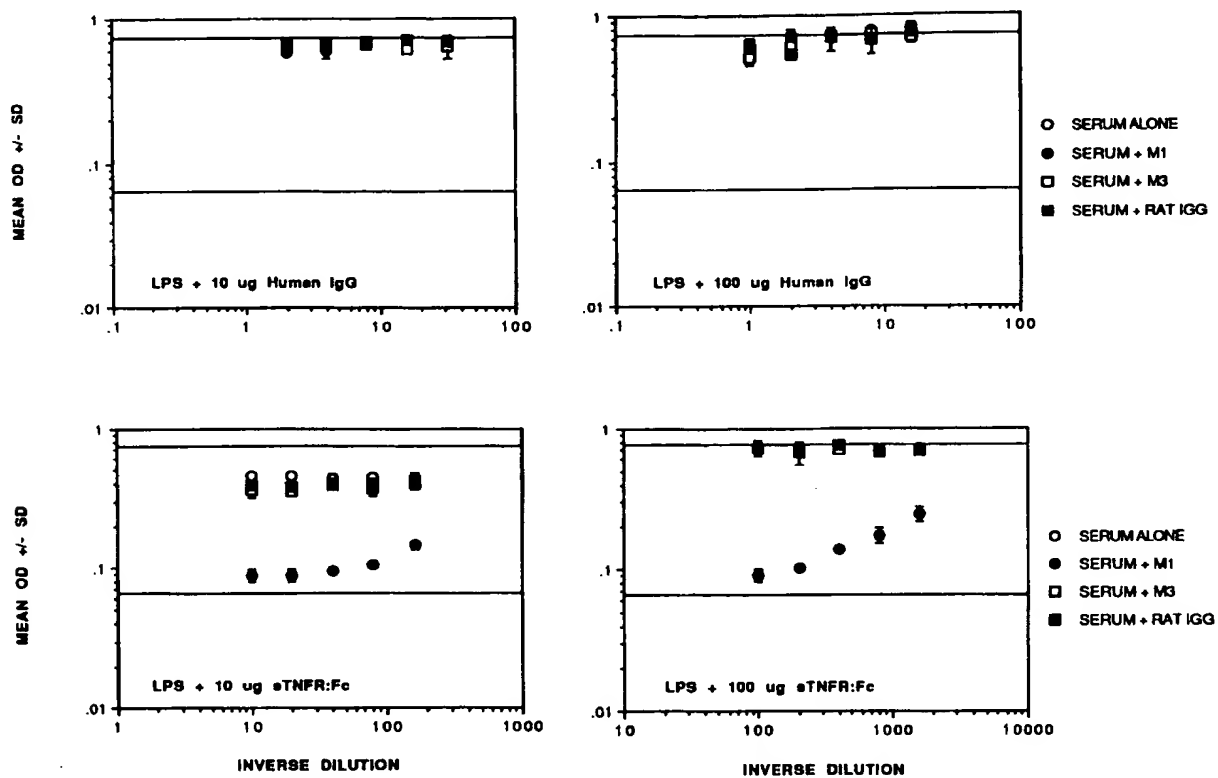


FIGURE 11. Prolongation of serum TNF in vivo by sTNFR:Fc. The protocol was identical to that described in the legend to Figure 10 except that the serum samples were obtained 4 h after LPS injection.

sTNFR:Fc molecules. If TNF is also released from the sTNFR:Fc molecule in vivo, the sTNFR:Fc molecules may function by dissipating the peak in serum TNF levels normally associated with bolus LPS injection.

Soluble TNFR:Fc molecules also function as carriers of TNF in that they alter the rate at which TNF disappears from the serum of LPS-treated mice. Control mice injected with LPS alone or LPS and human IgG had elevated serum TNF levels only during the first 2 h after injection (Figs. 6, 10, 11). However, mice treated with LPS and sTNFR:Fc retained TNF in their serum for at least 4 h (Fig. 11). In support of these data, we have demonstrated that the $t_{1/2\beta}$ of labeled TNF is increased approximately fourfold in vivo when injected concomitantly with sTNFR:Fc (D. Lynch and K. M. Mohler, unpublished observations). These results suggest that the sTNFR:Fc protein functions as an effective antagonist of LPS induced mortality by acting as a biologic buffer for TNF activity.

When mice were exposed to lethal doses of LPS and low doses of sTNFR, which failed to affect mortality incidence, serum TNF levels as detected in the L929 bioassay were elevated in comparison to control mice receiving LPS alone or LPS plus IgG (Figs. 7 and 8). However, despite the fact that low doses of sTNFR increased serum TNF activity, no agonistic activity in terms of mortality could be demonstrated when low doses of sTNFR were administered in

conjunction with an LD₅₀ dose of LPS (Fig. 12) (data not shown). These data indicate that the agonistic effects on serum TNF activity obtained in vivo in the presence of sTNFR were distinct from the effects of sTNFR on LPS-induced mortality. Alternatively, the sTNFR may function as an agonist only with lethal doses of LPS. If the latter hypothesis is correct, then lower (nonlethal) doses of LPS may induce sufficient quantities of endogenous soluble TNFR so that the administration of exogenous sTNFR:Fc molecules would have relatively minor additional biologic impact.

Several types of TNFR/antibody-based fusion proteins have been described and tested for efficacy in murine LPS-induced mortality models (38, 39). These TNF antagonists include the molecule employed in the present study, composed of the extracellular portion of the p80 cell-surface receptor linked to the Fc region of human IgG1, as well as molecules consisting of fusions between the extracellular portion of the p60 TNFR combined with the Fc region of either human IgG1 (38) or human IgG3 (39). The dose of p60 sTNFR:Fc (4 to 20 μ g) (38, 39) and the dose of p80 sTNFR:Fc (10 to 100 μ g) (Fig. 3) required to demonstrate efficacy are similar. However, efficacy of the different constructs was influenced substantially by the timing of administration relative to lethal LPS injection. The p80 sTNFR:Fc (human IgG1) construct was efficacious when

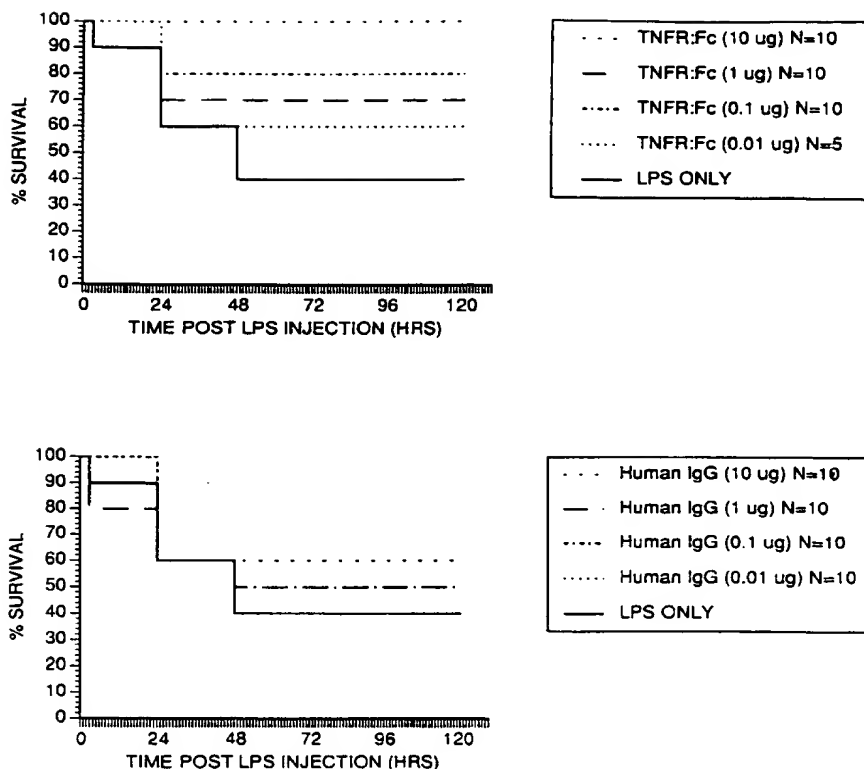


FIGURE 12. Administration of low doses of sTNFR:Fc is not detrimental to the host. BALB/c mice received an LD₅₀ dose of LPS (300 µg) premixed with low doses of sTNFR:Fc or human IgG. Survival was monitored at least once a day for 5 days.

administered as late as 3 h after LPS administration (Fig. 5). However, a sTNFR:Fc fusion protein consisting of the p60 sTNFR coupled to human IgG1 was effective only if administered within 1 h after lethal LPS injection (38). In contrast, preliminary reports utilizing the p60 sTNFR coupled with human IgG3 indicated that partial benefits were obtained as late as 3 h after LPS injection (39). Thus, significant differences exist between the published abilities of different sTNFR:Fc fusion proteins to function after LPS administration, and these differences do not appear to correlate with either the particular sTNFR (i.e., p60 or p80) or with the subclass of human IgG utilized for the fusion protein.

The relationship between serum TNF activity and efficacy of the sTNFR:Fc molecule had not been established before the present study. Given the ability of sTNFR:Fc to function effectively when administered as late as 3 h after LPS injection (Fig. 5), it was somewhat surprising to observe that the vast majority of detectable serum TNF activity had already passed by 3 h (Fig. 6). A number of hypotheses, which are not necessarily mutually exclusive, may explain these results. First, the length of time that TNF must be bound to its cell surface receptor prior to the induction of an irreversible biological effect such as cell lysis is unknown. However, studies by Engelberts et al. (40) suggest that TNF must be present for extended periods of time to achieve maximal biologic activity *in vitro*. Thus, sTNFR:Fc may be able to compete for TNF which has

already bound to the cell surface and, in effect, dislodge it before the interaction has occurred for a time sufficient to result in complete biologic signaling. In this regard, the rate of dissociation of radiolabeled TNF from its cell surface receptor *in vitro* is increased in the presence of either unlabeled TNF (41) or the dimeric sTNFR:Fc (C. Smith, unpublished results). Second, LPS-induced mortality may result from the cumulative effect of TNF. Thus, inhibition of the small amount of TNF present late in the time course might be sufficient to prevent mortality. Third, the therapeutic potential of the sTNFR:Fc molecule may not be related solely to the removal of serum TNF activity. The sTNFR:Fc molecule could function by inhibiting TNF activity in extravascular sites. Finally, LPS-induced toxicity may be mediated at least in part by TNF expressed on the cell surface, which may be masked in the presence of sTNFR:Fc. Regardless of the mechanism of efficacy of the sTNFR:Fc molecule, there is a relatively small window of time, 3 to 4 h after LPS injection, during which serum TNF levels are low and administration of the sTNFR:Fc molecule is still efficacious. These results also suggest that serum TNF levels may not always be a good prognostic indicator for the clinical efficacy of the sTNFR:Fc molecule.

Soluble TNF-binding proteins have been recovered from the urine of normal humans (42, 43) and appear at elevated levels in the serum of cancer patients (44, 45) and in response to endotoxin challenge (46). The biologic role of these TNF-binding proteins is currently under investiga-

tion. Previous investigators have demonstrated that TNF spontaneously loses activity in vitro and, under some circumstances, soluble p60 and p80 TNFR can prevent its spontaneous degradation, thereby enhancing the biological longevity of TNF (47). Our experiments demonstrated that a sTNFR monomer can function as an agonist of serum TNF activity in vivo and a sTNFR:Fc molecule could act either as an agonist or antagonist of serum TNF levels in a dose dependent fashion. Thus, the biologic effect of the soluble TNF-binding proteins isolated from humans will probably vary depending upon the relative concentration of TNF and sTNFR. This concept is supported by recent data of Girardin et al. (48), demonstrating increased concentrations of both TNF and soluble TNFR in the serum of septic patients. In that study, higher ratios of soluble TNFR to TNF correlated with increased probability of survival.

These experiments indicate that the sTNFR:Fc molecule is an effective antagonist of LPS-induced septic shock and are in agreement with a number of studies that have shown the beneficial effects of antagonizing TNF activity in sepsis with either antibody (1–3) or soluble receptors (38, 39). In aggregate these results indicate that TNF plays a central role in mediating the lethality associated with sepsis. However, several lines of evidence suggest that the role of cytokines in sepsis is not yet fully understood. First, antagonism of several cytokines other than TNF (e.g., IFN- γ and IL-1) can also lead to beneficial results (49, 50). Second, anti-TNF antibodies have been reported to have variable therapeutic potential in models of endotoxemia, cecal ligation and puncture and bacterial sepsis (51–53). Further experimentation will be required to determine whether or not the sTNFR:Fc molecule also displays the same spectrum of efficacy. However, the results presented here suggest that the sTNFR:Fc molecule may be a useful therapeutic agent for sepsis and other inflammatory diseases.

Acknowledgments

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results of BLAST

BLASTP 2.2.14 [May-07-2006]

Reference:

Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

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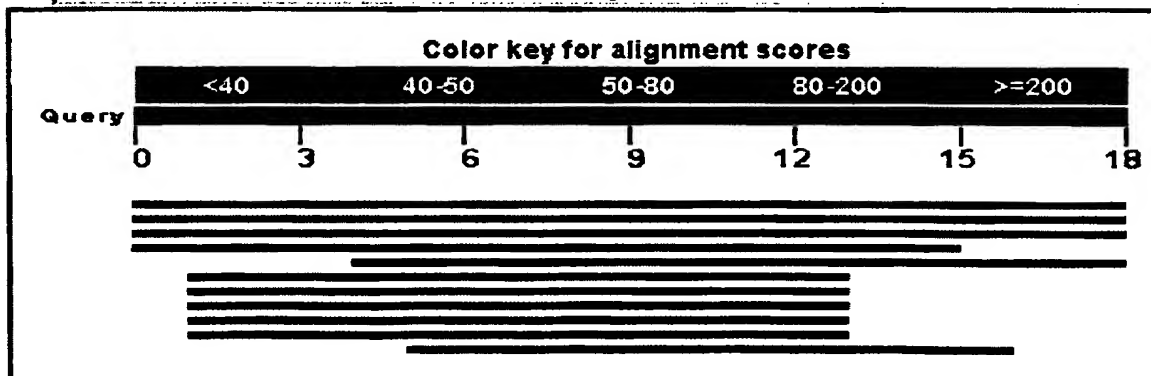
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Distribution of 11 Blast Hits on the Query Sequence

Mouse over to see the define, click to show alignments





[Tree view](#) NEW

Sequences producing significant alignments:

Score (Bits) E Value

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



Alignments

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construct]
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(Tumor necrosis factor receptor 2) (TNF-R2) (Tumor necrosis
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antigen) (Etanercept) [Contains: Tumor necrosis factor
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AF P Y PEPGS
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The Active Form of Tumor Necrosis Factor Is a Trimer*

(Received for publication, December 1, 1986)

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Natural human and recombinant human and murine tumor necrosis factors (TNF) were fractionated by gel filtration chromatography on Sephadex G-75. The active form of TNF was identified by its inhibitory activity in receptor binding assays with HeLa cells and was eluted as a protein of $M_r \sim 55,000$. Radioiodinated human and murine TNF were fractionated by gel filtration into a major peak of $M_r \sim 55,000$, corresponding to a trimer, and a minor peak of $M_r \sim 17,000$, corresponding to a monomer. Binding assays showed that the trimer was at least 8-fold more active than the monomer. The human TNF partially dissociated into monomers upon addition of the nonionic detergent Triton X-100. Isolated monomers showed low binding affinity ($K_D = 70$ nM) and reduced cytotoxicity, whereas trimers showed high binding affinity ($K_D = 90$ pM) and cytotoxicity. When ^{125}I -TNF was bound to cells, no release of monomer was detectable, suggesting that the trimer could directly bind to cellular receptors without dissociating into subunits. Further evidence for such binding was obtained by cross-linking ^{125}I -TNF trimers with bis[2-(succinimidocarbonyloxy)ethyl]sulfone. These trimers were bound to HeLa cells, could be dissociated from cellular receptors, and elicited a cytotoxic response. These results show that trimers, whether native or cross-linked, bind to receptors and are the biologically active form of TNF.

Recombinant or natural human tumor necrosis factor (hTNF)¹ purified from tissue culture supernatants or serum is under denaturing conditions a polypeptide of M_r 17,000 (1) or 17,500 (2), respectively. However, the biological activity of TNF has been recovered under nondenaturing conditions in proteins thought to consist of dimers or higher oligomers with M_r values of 45,000 (1, 3) and 70,000 (4) for human TNF; 35,000 (5), >70,000 (6), 150,000 (7), 70,000 (8), and 55,000 (8, 9) for murine TNF; and 39,000 (10) or 55,000 (11) for rabbit TNF.

Numerous reports demonstrate that TNF interacts with

cellular receptors (2, 12-14) and elicits cytotoxic (15, 16) or growth regulatory responses (17, 18). It is not known, however, which form of TNF interacts with receptors. The goal of the present work was to examine whether hTNF and mTNF are under physiological conditions oligomers of defined size and to establish whether such oligomers directly bind to receptors. We report in this communication that TNF trimers bind to cellular receptors and elicit a cytotoxic response.

MATERIALS AND METHODS

Cytotoxicity Assay—HeLa S2 cells were grown in monolayer cultures in Dulbecco's medium supplemented with 10% heat-inactivated horse serum. For each assay, 4×10^5 cells were resuspended in 0.2 ml of culture medium containing 5 $\mu\text{g}/\text{ml}$ cycloheximide and the indicated concentrations of hTNF. After 18 h, the medium containing dead cells was removed and adherent cells were stained with 0.2% crystal violet in 2% ethanol (19). The dye was solubilized with 33% acetic acid, and the A_{540} was measured with a Titertek Multiscan (Flow Laboratories). Cytotoxicity was expressed as a percentage of the A_{540} of control cells that received cycloheximide alone.

Iodination and Cross-linking—Recombinant hTNF and mTNF were radioiodinated using a solid-phase lactoperoxidase procedure (20) to a specific activity of 10-58 Ci/g. ^{125}I -hTNF was cross-linked with the bifunctional reagent BSOCES (Pierce Chemical Co.); 209 ng of ^{125}I -hTNF in 0.1 ml of 75 mM sodium phosphate buffer, pH 7.5, containing 0.05% BSA were reacted with 1 mM BSOCES. After 10 min at 4 °C, 0.01 ml of 1 M glycine in 0.1 M sodium phosphate buffer, pH 7.5, was added. 0.1-ml samples were applied to a Sephadex G-75 column (0.7 \times 24 cm) equilibrated with 10 mM PBS, 0.1% BSA, and 0.15-0.20-ml fractions were collected.

Binding Assays—In competitive binding experiments, 0.3-0.5 ng of radioligand were incubated 5 h at 4 °C with 1×10^6 cells in 0.15 ml of medium containing 5 mM MgCl_2 and 40 mM HEPES, pH 7.5, as previously described (20). In experiments designed to recover cell-bound radioligand, the binding assays were proportionately increased 10-fold to 1.5 ml. Following binding, the cells were centrifuged at 4 °C and washed twice with 1 ml of PBS. To dissociate TNF-receptor complexes, 5 μl of 6 M GdnHCl, 0.1 M sodium phosphate, pH 7.5, were added to 10^7 pelleted cells for 10 min at 4 °C. Lysed cells were diluted with 0.1 ml of 10 mM PBS, 0.1% BSA and centrifuged for 10 min at $15,500 \times g$. The supernatants were chromatographed on Sephadex G-75 columns. Bindability was determined by incubating radioligands with graded amounts of excess cellular receptors, and the results were expressed as the maximum percentage of counts added that were specifically bound to HeLa S2 cells at 4 °C.

Zonal Centrifugation Studies—0.3 ng of ^{125}I -hTNF and 0.5 mg of reference protein in 0.1 ml of PBS were layered on 5-20% linear sucrose gradients. The gradients were centrifuged for 24 h at 44,000 rpm in an SW 50 rotor at 5 °C, and fractions containing 4 drops were collected. Ovalbumin ($M_r = 45,000$) and BSA ($M_r = 66,000$) were used as reference proteins.

RESULTS

Gel filtration on Sephadex G-75 was used to determine the M_r value of native TNF and to establish whether iodination altered its size. Accordingly, the elution profiles of native hTNF and mTNF were compared with those of ^{125}I -hTNF and ^{125}I -mTNF (Fig. 1). Since small amounts of TNF were chromatographed in the presence of carrier protein, the TNF was localized by its inhibitory activity in receptor binding assays. Both recombinant TNF eluted as a single major peak (Fig. 1A). The elution profile of natural hTNF (a gift of Dr. Walter Fiers, University of Ghent) was indistinguishable from that of recombinant hTNF (data not shown). Radioiodinated hTNF and mTNF eluted as a major peak (#1) in corresponding fractions, followed by a minor peak (#2) and free ^{125}I (Fig.

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† Supported by a postdoctoral fellowship of the Cancer Research Institute, New York.

¹ The abbreviations used are: hTNF, human tumor necrosis factor; mTNF, murine TNF; BSA, bovine serum albumin; PBS, phosphate-buffered saline; GdnHCl, guanidine hydrochloride; BSOCES, bis[2-(succinimidocarbonyloxy)ethyl]sulfone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

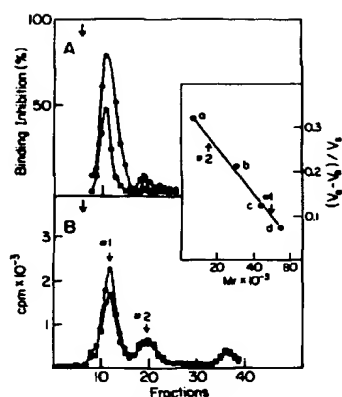


FIG. 1. Gel filtration on Sephadex G-75 of native hTNF and mTNF. A, 100 ng of hTNF (●) or mTNF (■) in 0.1 ml were chromatographed as described under "Materials and Methods." Aliquots of each fraction were assayed for inhibition of ^{125}I -hTNF binding on HeLa cells. B, 0.1-ml samples containing 15,000 cpm of ^{125}I -hTNF (●) or ^{125}I -mTNF (■) were chromatographed on the same column and the fractions were counted in a gamma counter. The void volume is indicated by an arrow. The inset shows the elution positions of peaks #1 and #2 with respect to proteins of known molecular size: a, cytochrome c; b, carbonic anhydrase; c, ovalbumin; and d, bovine serum albumin.

1B). Comparison of the elution volumes of peaks 1 and 2 with those of proteins of known size (Fig. 1, inset) gave M_r ~ 55,000 and 17,000, respectively, corresponding to a trimer and a monomer. This interpretation was confirmed by zonal sedimentation studies using ^{125}I -hTNF in isokinetic 5–20% sucrose gradients (21). The major peak sedimented as a globular protein of $M_r = 54,650 \pm 2,340$ in three independent analyses.

The TNF in the monomer peak appeared to compete poorly in the receptor binding assay, since it was not detected in the chromatogram shown in Fig. 1A. To confirm this finding, ^{125}I -hTNF trimer and small amounts of monomer were isolated by gel filtration. 10,000 cpm of each fraction were tested for binding to HeLa cells; $1,180 \pm 90$ cpm of trimer were bound in a standard assay, whereas only 140 ± 20 cpm of monomer were specifically bound. In subsequent experiments, the biological activity of trimer and monomer was compared in cytotoxicity assays. Since monomer was recovered in relatively small amounts in physiological solutions, different procedures were tried to obtain sufficient quantities of this species. It was thus found by gel filtration analysis that ^{125}I -hTNF trimer partially dissociated upon addition of low concentrations of the nonionic detergent Triton X-100. This dissociation was dependent on hTNF concentration, since the monomer/trimer ratio increased at low hTNF concentration (Fig. 2). Monomers were separated from trimers by gel filtration in a Sephadex G-75 column (0.7 × 24 cm) pre-equilibrated with either PBS, 0.1% BSA for binding assays or culture medium for cytotoxicity assays. Triton X-100 was not detected (22) in the trimer or monomer peak, but was eluted at a greater column volume than hTNF monomer. In separate experiments (data not presented), it was shown by gel filtration analysis that hTNF monomers (~1 ng/ml) prepared in this manner quantitatively reassociated to trimers when the concentration of hTNF was increased 500-fold by adding unlabeled hTNF. Therefore, hTNF trimers can be dissociated by the addition of Triton X-100 into monomers, which are relatively stable in dilute solutions but readily reassociate to trimers when the hTNF concentration is raised.

Pooled fractions of hTNF trimer and monomer were subsequently compared in competitive binding and cytotoxicity assays on an equal counts/min basis. The monomer fraction

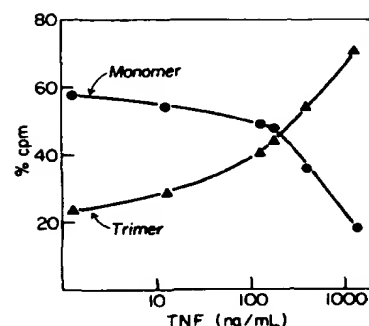


FIG. 2. Concentration dependence of the dissociation of ^{125}I -hTNF by 0.1% Triton X-100. Increasing quantities of ^{125}I -hTNF were incubated for 10 min at 22 °C in a total volume of 0.1 ml containing 0.1% Triton X-100, 0.1% BSA, 20 mM PBS, pH 7.5, and were applied to a Sephadex G-75 column (0.7 × 24 cm) equilibrated at 4 °C with PBS, 0.1% BSA. 0.15-ml fractions were collected. The trimer and monomer recovered are expressed as a ratio of the counts/min in the respective peaks.

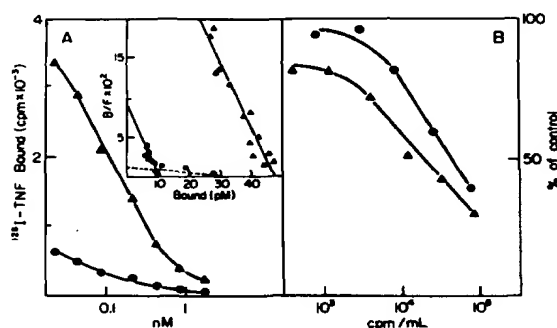


FIG. 3. Competitive binding and cytotoxicity assays comparing ^{125}I -hTNF monomer and trimer fractions. Trimer (▲) and monomer (●) fractions were isolated by gel filtration as described in the legend to Fig. 2. A, 30,000 cpm of each fraction were incubated for 5 h at 4 °C with 10^6 HeLa cells and various concentrations of unlabeled hTNF in a total volume of 0.15 ml (see "Materials and Methods"). B, 8×10^4 HeLa S2 cells were grown in monolayers in 96-well plates with 5 $\mu\text{g}/\text{ml}$ cycloheximide and the indicated cpm/ml of TNF monomer (●) or trimer (▲). After 18 h the culture medium containing dead cells was removed, and the adherent cells were stained as described under "Materials and Methods." Results are expressed as a percentage of the control cells incubated with cycloheximide alone. Inset, binding data are shown as Scatchard plots with nonspecific binding subtracted; 51,300 was used as the molecular weight for TNF.

showed low binding activity and cytotoxicity compared to the trimer fraction. Binding of monomer was about 5.5-fold lower than that of trimer, as determined in competition binding assays (Fig. 3A). Scatchard plots of these data showed that the trimer was bound with a $K_D = 90$ pM, whereas only a small component of the monomer fraction was bound with such high affinity (Fig. 3A, inset). Most of the monomer was bound with low affinity ($K_D = 70$ nM). It seems unlikely that this binding has biological relevance, since 50% cytotoxicity of HeLa cells is observed with 2 pM hTNF (20). In parallel cytotoxicity assays, a monomer concentration 6–7-fold greater than that of trimer was needed to elicit the same biological response when tested at low concentrations (Fig. 3B). However, at the highest concentrations tested, the cytotoxicity of monomer was nearly equivalent to that of trimer. Rechromatography of the monomer fraction at the end of the incubation period showed the presence of about 10% trimer (data not shown), which could account for both the small component binding with high affinity and for the cytotoxicity at the

highest concentrations tested. These results indicated that hTNF trimer binds with higher affinity to receptors and has greater cytotoxic activity than hTNF monomer.

In the following experiment, we examined whether hTNF dissociates into monomers upon binding to receptors. A binding assay was carried out at 4 °C with low ^{125}I -hTNF concentration, and the supernatant obtained after spinning out the cells was analyzed by gel filtration. The trimer peak was reduced in proportion to the ^{125}I -hTNF bound to the cells, but no increase in the monomer peak could be detected (Fig. 4A). This result suggested that hTNF trimers could directly bind to receptors, but it could not be excluded that monomers

or dimers were binding and that the subunits released were reassociating into trimers. Therefore, to demonstrate that trimers can bind to cells and have biological activity, the hTNF was cross-linked to prevent its dissociation.

The ^{125}I -hTNF was reacted with the cross-linking reagent BSOCOES and compared to control ^{125}I -hTNF by gel filtration chromatography, binding to HeLa cell receptors, and cytotoxicity assays. The cross-linked ^{125}I -hTNF eluted with $M_r \sim 55,000$ even after treatment with 3 M GdnHCl (Fig. 4B). This demonstrated that cross-linking stabilized hTNF against dissociation. In contrast, 85% of the control ^{125}I -hTNF treated with 3 M GdnHCl eluted with an $M_r = 17,000$ (Fig. 4C). This dissociation was in large part reversible, since after dialysis ^{125}I -hTNF eluted as a trimer (Fig. 4D). These experiments showed that hTNF cross-linked with BSOCOES is a trimer resistant to dissociation by relatively strong denaturing reagents, such as GdnHCl. However, drastic denaturing treatment of the cross-linked trimer, such as boiling in 1% sodium dodecyl sulfate under reducing conditions, resulted in partial dissociation into dimers and monomers, as judged by gel electrophoresis (Fig. 4, inset).

A binding assay carried out with cross-linked hTNF showed that it could bind to TNF receptors of HeLa cells. These cells were treated with 3 M GdnHCl to release bound BSOCOES- ^{125}I -hTNF, and the supernatant was analyzed by gel filtration. A single peak of radioactivity was present (Fig. 4E), demonstrating that the bound hTNF could be eluted from HeLa cell receptors as a trimer. In order to determine whether cross-linking or acylation of amino groups had altered its binding to cell receptors, BSOCOES- ^{125}I -hTNF was compared with ^{125}I -hTNF in competitive binding assays (Fig. 5). Since BSOCOES- ^{125}I -hTNF had lower bindability (39% compared to 45% for ^{125}I -hTNF), equivalent amounts of bindable radioligands were added. The binding of both ligands was inhibited in a parallel manner by unlabeled hTNF, but only half as much unlabeled hTNF was required for 50% competition of BSOCOES- ^{125}I -hTNF. This indicated that chemical changes introduced by the cross-linking reagent resulted in partial loss of binding activity. However, binding was 90% specific for both ligands. In agreement with the loss of binding activity, the cytotoxicity of BSOCOES- ^{125}I -hTNF for HeLa cells was on average 4.5-fold less than that of control ^{125}I -hTNF. A similar 5–10-fold decrease in cytotoxicity of BSOCOES- ^{125}I -hTNF was observed in experiments with SK-MEL-109 melanoma cells (data not shown). These results with cross-linked TNF confirmed the findings with native TNF by showing that stable TNF trimers bind to cellular receptors and elicit a biological response.

DISCUSSION

Natural human TNF, recombinant hTNF and mTNF, and cross-linked ^{125}I -hTNF coelute in gel filtration under non-denaturing conditions as a major peak with an apparent $M_r \sim 55,000$ (Figs. 1 and 4). The formation of homotrimers from 17,100 monomers (1) gives a predicted $M_r = 51,300$, which is in fairly good agreement with the M_r value obtained from gel filtration or zonal sedimentation. Furthermore, after cross-linking ^{125}I -hTNF with BSOCOES, radioactive bands corresponding to trimers, dimers, and monomers are observed by gel electrophoresis. Cross-linking data for other oligomeric proteins similarly show that incompletely cross-linked homotrimers may be dissociated into monomers and dimers (23). Therefore, three lines of evidence indicate that natural and recombinant TNF exist predominantly as a trimer under physiological conditions.

Gel filtration analyses show that monomers are present as

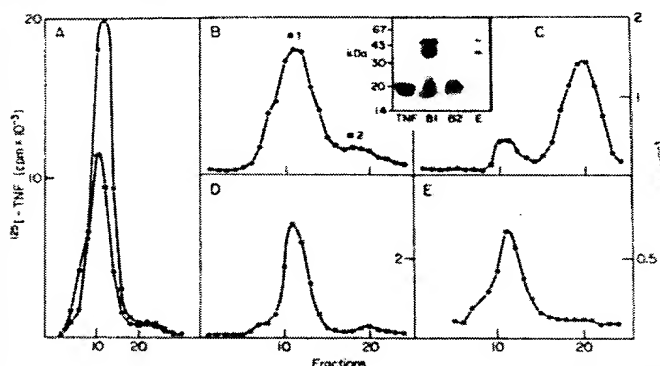


FIG. 4. Gel filtration of native and cross-linked ^{125}I -hTNF on Sephadex G-75. A, 0.2-ml aliquots of pre-binding (■) and post-binding (●) cell-free supernatants containing 91,460 and 63,960 cpm, respectively, were compared by gel filtration in a Sephadex G-75 column (0.7 × 25 cm). 10,000 cpm of ^{125}I -hTNF were incubated per 10^6 HeLa cells for 4 h at 4 °C. The cells were centrifuged at $15,500 \times g$ for 30 s, and the supernatant was immediately applied to the column. The difference in peak areas represents TNF bound to cells. B–E, gel filtration in a different Sephadex G-75 column (0.7 × 24 cm). B and C, 20,000 cpm of BSOCOES- ^{125}I -hTNF (B) or ^{125}I -hTNF (C) in 5 μl were mixed with an equal volume of 6 M GdnHCl. After 10 min, 85 μl of column buffer and 5 μl of glycerol were added, and the sample was applied to the column. D, 40 μl of ^{125}I -hTNF in PBS, 0.1% BSA were mixed with 40 μl of 6 M GdnHCl and after 10 min at room temperature was dialyzed for 24 h at 4 °C against PBS, 0.1% BSA and 20 μM 2-mercaptoethanol before chromatography. E, 4×10^6 HeLa cells with bound BSOCOES- ^{125}I -hTNF (6,750 cpm) were washed with PBS and then treated with 10 μl of 3 M GdnHCl to dissociate bound TNF. After 10-min centrifugation at $15,500 \times g$ to remove cellular debris, 0.1 ml of supernatant containing 2,800 cpm and 5% glycerol was applied to the column. The inset shows an electrophoretic analysis of ^{125}I -hTNF (monomer) and of peak fractions recovered from the chromatograms in B and E containing cross-linked trimers and dimers. M_r markers are indicated on the left.

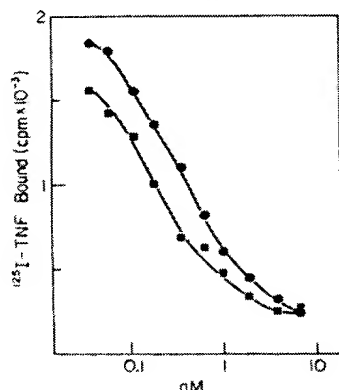


FIG. 5. Competitive binding assays comparing cross-linked and native hTNF. 18,000 cpm of ^{125}I -hTNF (●) or BSOCOES- ^{125}I -hTNF (■) were incubated with 10^6 HeLa cells and unlabeled hTNF as described under "Materials and Methods."

a small component of radioiodinated TNF (Fig. 1). The isolated monomers are only 12% as active as trimers in binding assays. Furthermore, ^{125}I -hTNF can be dissociated into monomers by several treatments, such as a short incubation at pH 3.0.² Several reports have indicated that the biological activity of TNF is pH-sensitive (6, 9, 11, 24). Treatment with 3 M GdnHCl also dissociates ^{125}I -hTNF (Fig. 4C). Of particular interest is the finding that low concentrations of the nonionic detergent Triton X-100 partially dissociate hTNF into monomers (Fig. 2), suggesting that weak hydrophobic interactions may be responsible for stabilizing the trimers (25). Dissociation by Triton X-100 can be used in combination with gel filtration chromatography to obtain monomer and trimer fractions for competitive binding and cytotoxicity assays.

These ^{125}I -hTNF monomers show low receptor binding activity when compared to trimers. Monomer binding is characterized by high and low affinity components. The high affinity component is the same as that observed for the trimer, but rechromatography of monomer fractions shows the presence of some trimers. Therefore, small amounts of contaminating trimers may account for the high affinity binding component. In contrast, the trimer exhibits a single high affinity binding and greater cytotoxicity than monomer. At low concentrations, the cytotoxicity of the monomer fraction is 6-7-fold less than that of the trimer, but at higher concentrations monomer cytotoxicity becomes equivalent to that of trimer. This finding may be explained by the reassociation of monomers into active trimers. Other reports suggest that TNF monomers and oligomers are all active (1, 4, 12, 26).

The most direct evidence that hTNF trimer is biologically active comes from experiments wherein ^{125}I -hTNF is cross-linked with BSOCOES. The cross-linked hTNF binds to receptors (Fig. 5) and is cytotoxic. Moreover, cross-linked hTNF trimers are recovered after binding to cells (Fig. 4E). In view of the remarkably low concentrations of hTNF that are biologically active in cytotoxicity assays (27), we are lead to speculate that these trimers may interact simultaneously or sequentially with more than one receptor. A possible result of such multiple interactions may be a heightened effective concentration at the cell surface (28). Furthermore, simultaneous binding to neighboring receptors might favor the interaction of the cytoplasmic domain of receptors (29) and either trigger or amplify the as-yet unknown signaling mechanism of the TNF receptor. In addition, dissociation of TNF into monomers at low concentrations may have some physiological relevance in the action of this factor. Since the monomer appears to be less active than the trimer, this dissociation may limit some of the deleterious effects of TNF (30) at sites remote from those where it is produced in high amounts by macrophages (31).

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² R. A. Smith and C. Baglioni, unpublished observations.

Multimeric Structure of the Tumor Necrosis Factor Receptor of HeLa Cells*

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The tumor necrosis factor (TNF) receptor of HeLa cells was solubilized in Triton X-100 and characterized by gel filtration, affinity labeling, and ligand blotting studies. Receptors solubilized with Triton X-100 eluted in gel filtration as a major peak of $M_r = 330,000$ and retained high affinity binding ($K_D = 0.25$ nM). Affinity labeling of soluble receptor/ ^{125}I -TNF complexes using the reversible, bifunctional bis[2-(succinimidocarbonyloxy)ethyl]sulfone resulted in the formation of cross-linked species of $M_r = 310,000$, 150,000–175,000, 95,000, and 75,000. The formation of these complexes was competitively inhibited by unlabeled TNF. Partial reversal of cross-linking in these complexes and their analysis by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) resolved ^{125}I -TNF dimers cleaved from the 95,000 band and ^{125}I -TNF monomer cleaved from the 75,000 band, providing evidence for a $M_r \sim 60,000$ subunit. In addition, the 95,000 and 75,000 bands were resolved as components of larger complexes ($M_r = 150,000$ –175,000), which presumably contain two receptor subunits. The M_r 95,000 and 75,000 bands were also released from the M_r 310,000 complex by reduction with dithiothreitol, suggesting a role for disulfide bond stabilization. To investigate the association of the putative receptor subunits, Triton X-100 extracts from HeLa membranes were fractionated by SDS-PAGE without reduction and transferred electrophoretically to nylon membranes for TNF binding assays. Only two bands of $M_r = 60,000$ and 70,000 specifically bound TNF, and higher M_r binding activity was not observed. These results indicate that TNF receptors in HeLa cells are high molecular weight complexes containing $M_r = 60,000$ and 70,000 subunits each capable of binding TNF and that the complexes are primarily stabilized by non-covalent, hydrophobic interactions.

Tumor necrosis factor (TNF)¹ was initially identified as a mediator causing hemorrhagic necrosis of certain transplantable tumors in mice that had been primed with bacillus

Calmette-Guérin and subsequently challenged with endotoxin (1). Studies in different cell lines have identified diverse actions for this cytokine including inhibition of lipoprotein lipase in adipocytes (2), induction of class I major histocompatibility antigens in endothelial cells and fibroblasts (3), growth stimulation of fibroblasts (4, 5), increased expression of epidermal growth factor receptors in fibroblasts (6), proliferation and activation of the *c-myc* oncogene in osteosarcoma cells (7), and bone resorption in osteoclasts (8, 9). TNF induces synthesis of plasminogen activator inhibitors (10), superoxide dismutase (11), and production of other cytokines, such as interleukin-1, macrophage colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor (12). These and other cellular responses indicate that TNF has a major role as a mediator of inflammation (13) and endotoxic shock (14).

Cellular responses to TNF are mediated through binding of TNF to specific cell surface receptors (15). Human TNF forms stable trimers in solution (16, 17) that contain identical polypeptides of M_r 17,350 (18). The trimeric form of TNF binds to receptors (19) that are characterized by a single class of high affinity binding sites (20, 21). After binding to receptors, TNF is internalized and degraded (22–24).

Structural information about the TNF receptor has been provided by affinity labeling studies (25–30). These data are difficult to interpret in terms of receptor structure because cross-linked TNF itself is resolved by SDS-PAGE into trimers, dimers, and monomers (19), leaving the interpretation of cross-linked TNF-receptor complexes equivocal (20). In this report are presented the results of studies on the structure of TNF receptors of HeLa cells. Cell membranes were solubilized with detergent and the relative molecular mass (M_r) of soluble receptor preparations was determined by gel filtration chromatography. To investigate the receptor structure, cross-linked TNF was used in combination with a reversible cross-linking agent to detect TNF monomers, dimers, and trimers bound to receptor subunits. A possible role for disulfide bonds in stabilizing complexes of M_r 310,000 was investigated. Evidence is presented that receptors contain two polypeptides that bind TNF.

MATERIALS AND METHODS

Preparation and Solubilization of Cell Membranes—HeLa S2 cells were grown to a density of $5\text{--}7 \times 10^6$ cells/ml in spinner cultures in Joklik's medium (GIBCO) with 7% horse serum. The cells were collected and washed in cold phosphate-buffered saline by centrifugation at $300 \times g$. In subsequent steps performed at $0\text{--}4^\circ\text{C}$, 10^8 cells were suspended for 10 min in 5 ml of hypotonic phosphate-buffered saline (PBS diluted 1:20) and broken with a Dounce homogenizer; 5 ml of 0.5 M sucrose, 2 mM EDTA, 0.004% 2-mercaptoethanol, and 40 mM HEPES buffer, pH 7.5, were added. Concentrated protease inhibitors were added to homogenates to obtain a final concentration of 5 $\mu\text{g/ml}$ pepstatin A, 50 $\mu\text{g/ml}$ aprotinin, 0.1 mM benzamidin, and 0.1 mM phenylmethylsulfonyl fluoride. Following a centrifugation at

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¹The abbreviations used are: TNF, human tumor necrosis factor; BSOE, bis[2-(succinimidocarbonyloxy)ethyl]sulfone; DSS, disuccinimidyl suberate; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; bio-TNF, biotinylated-TNF.

1,500 \times g for 10 min to remove unbroken cells and nuclei, supernatants were centrifuged at 20,000 \times g for 60 min over 10 ml of 35% (w/w) sucrose in 20 mM HEPES, pH 7.5, containing protease inhibitors. The membranes retained at the interface were diluted 10-fold in this buffer without sucrose and were centrifuged again at 27,000 \times g for 30 min. The pellets obtained were resuspended in 10% glycerol, 2 mM MgCl_2 , 20 mM HEPES, pH 7.5, at a protein concentration of 8–12 mg/ml and stored at -60°C . To solubilize the membranes, we added 0.5 g of Triton X-100/g of protein and protease inhibitors, as indicated above. After 10 min, insoluble material was removed by centrifugation at 150,000 \times g for 30 min.

Gel Filtration.—An Ultrogel Aca34 column (1.6 \times 87 cm) was equilibrated at 4°C with 0.1% Triton X-100, 5 mM MgCl_2 , 50 mM Tris, pH 7.5. Samples of solubilized membranes or reference proteins were applied to the column in 1 ml. The following proteins with molecular weights (M_r) in parenthesis were used to calibrate the column: apoferritin (443,000), β -amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000) and carbonic anhydrase (29,000).

Biotinylation and Iodination of TNF.—TNF was biotinylated and radiolabeled in the same reaction vessel. Briefly, 10 μg of TNF in 50 μl of 0.1 M sodium phosphate buffer, pH 7.5, was reacted with 0.1 mM sulfo-succinimidyl 6-(biotinamido) hexanoate (Pierce Chemical Co.) for 30 min at 4°C . The biotinylation was quenched by addition of glycine to 10 mM. For radiolabeling of either TNF or biotinylated TNF (bio-TNF), iodinations were carried out as previously described (25). 10 μg of TNF was reacted with 2 mCi of Na^{125}I using a solid-phase lactoperoxidase procedure, and radioligands were purified by gel filtration.

Radioligand Binding Assays.—Either ^{125}I -TNF or ^{125}I -bio-TNF was bound to HeLa cells using the procedures previously described (25) or as indicated in figure legends. For binding in membrane assays, 1 ng of ^{125}I -TNF was incubated for 5–10 h at 4°C with an aliquot of membrane suspension (0.1 mg of protein) in 0.1 ml of 0.1 M sucrose, 5 mM MgCl_2 , 0.1% bovine serum albumin, and 25 mM HEPES buffer, pH 7.5. Membrane incubates were centrifuged at 15,000 \times g for 10 min, and supernatants were removed. For soluble binding assays, 1–3 ng of ^{125}I -TNF was incubated at 4°C with 0.05–0.2 ml of soluble receptor preparation in 0.1–0.3-ml reactions containing 25 mM HEPES buffer, pH 7.5, 5 mM MgCl_2 , 0.1% bovine serum albumin and 0.1% Triton X-100. After 8–18 h, 0.4 mg bovine γ -globulin (Sigma) and polyethylene glycol 6000 (PEG; final concentration 8%) were added to each incubation. Precipitates were allowed to form for 10 min at 4°C before centrifugation at 15,000 \times g for 10 min. The PEG-precipitates and membrane pellets were counted in a gamma counter. Specific binding was determined by subtracting counts precipitated in the presence of 100-fold excess unlabeled TNF.

Cross-linking and Gel Electrophoresis.—BSOCOES and disuccinimidyl suberate (DSS, Pierce Chemical Co.) were prepared as 100-fold concentrated solutions in dimethyl sulfoxide and added at the final concentrations indicated to samples buffered with 50 mM HEPES or 0.1 M sodium phosphate buffer, pH 7.5. After 10 min at 4°C , glycine in 0.1 M sodium phosphate buffer, pH 7.5, was added to a final concentration of 0.1 M, and the reactions were continued for an additional 10 min. SDS-PAGE (31) was performed in 4–8% linear gradient gels, unless otherwise indicated. Before first dimension electrophoresis, cross-linked proteins were heated at 100°C for 3 min in 2% SDS, 10% glycerol, and 60 mM Tris, pH 6.8 (sample buffer), with or without 50 mM dithiothreitol (DTT). Before second dimension electrophoresis, excised lanes from first dimension gels were incubated for 1 h at 37°C in buffer containing 0.1% SDS, 2 mM dithiothreitol, 6 M urea, 0.125 M Tris, pH 11.6, to reverse BSOCOES cross-linking (32). These acrylamide strips were then equilibrated in sample buffer for 1 h at 37°C before the second dimension SDS-PAGE. Materials for SDS-PAGE including reference proteins were from Bio-Rad.

Transfer to Nylon Membrane and Ligand Blotting.—0.4 mg of protein solubilized with Triton X-100 from membranes were applied per lane in buffer containing 1% SDS, 10% (w/v) glycerol, and 60 mM Tris, pH 6.8, at 4°C . SDS-PAGE was at a constant current of 25 mA/gel and 4°C in 4–8% gradient gels. Electrophoretic transfer was at 4°C for 3 h starting at 0.4 amp as described (33). Individual strips of nylon membrane (GeneScreen Plus, Du Pont) corresponding to lanes during SDS-PAGE were incubated with 1% hemoglobin in phosphate-buffered saline before addition of 10 ng of ^{125}I -TNF/ml for 4 h at 4°C in the absence or presence of 100-fold excess unlabeled TNF. Prestained reference proteins (Bethesda Research Laboratories) were also transferred.

RESULTS

Solubilization and Fractionation of TNF Receptors.—HeLa cell membranes were solubilized with Triton X-100, and conditions were found which yielded an optimal recovery of TNF binding activity. The ^{125}I -TNF-receptor complexes formed in incubations with solubilized membranes were coprecipitated with bovine γ -globulin using different concentrations of PEG. By varying the concentration of both Triton X-100 and PEG, optimal conditions for assay of TNF binding activity were determined. The highest TNF binding activity was recovered using 0.5 g of Triton X-100/g of membrane protein. Concentrations of Triton X-100 greater than 0.1% in the binding assay had an inhibitory effect. Specific binding at 4°C in this assay reached a plateau after 12 h and was stable up to 22 h. Nonspecific coprecipitation of TNF with bovine γ -globulin and PEG ranged from 20–60% and was subtracted to arrive at specific binding. The K_D in Triton X-100 extracts from HeLa membranes ranged from 300 to 1400 pM with 150–200 fmol of receptor/mg protein. This compared favorably with HeLa membranes which reached maximum binding at 4°C after 8 h, exhibited K_D ranging from 50 to 350 pM, and contained 50–80 fmol of receptor/mg protein. Solubilized membrane proteins were fractionated by gel filtration (Fig. 1). The binding activity eluted from an Aca 34 Ultrogel column in a main peak of $M_r = 330,000$, followed by two shoulders of $M_r = 175,000$ and 90,000, when compared with globular reference proteins. To determine if the M_r 330,000 fraction bound TNF with high affinity, aliquots from this peak were tested in competitive binding assays (Fig. 2). Analysis of the binding data using the curve-fitting LIGAND program (34) and Scatchard plot (*inset*) were consistent with a binding constant $K_D = 0.25$ nM and 1.5 pmol of receptor/mg protein. These results indicated that high affinity receptors eluted in the M_r 330,000 peak.

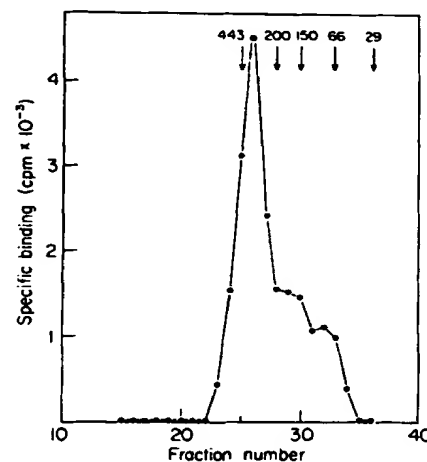


FIG. 1. Gel filtration of Triton X-100 extract from HeLa membranes. HeLa cell membranes (22 mg) prepared as described under "Materials and Methods" were solubilized with 0.5% Triton X-100 and applied to an Ultrogel Aca34 column (1.5 \times 87 cm) equilibrated at 4°C with 0.1% Triton X-100, 5 mM MgCl_2 and 50 mM Tris buffer, pH 7.5. Duplicate 0.2-ml aliquots from the column fractions (3.1 ml) were monitored for TNF binding using the PEG precipitation assay in the presence and absence of 100-fold excess unlabeled TNF. Nonspecific binding was 20% of the total counts added (40,000). These results are representative of multiple column runs. Globular proteins of known M_r were used to calibrate the column: A, apoferritin; B, β -amylase; C, alcohol dehydrogenase; D, bovine serum albumin; and E, carbonic anhydrase. The molecular mass is indicated in kDa above the arrows that show the peak elution fraction for each marker protein.

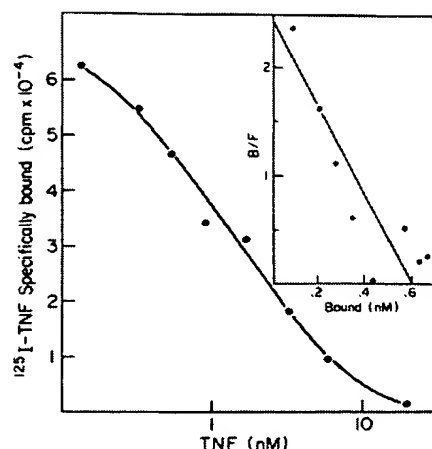


FIG. 2. Competitive binding in the M_r gel filtration peak. Aliquots containing 40 μ g of protein from the M_r 330,000 peak tube (see Fig. 1) were incubated 18 h at 4 °C with 3 ng of 125 I-TNF (240,000 cpm) in the presence or absence of the indicated concentrations of unlabeled TNF. The incubates were precipitated by the addition of 0.4 mg of bovine γ -globulin and PEG to a final concentration of 8%. Scatchard plot (inset) and analysis of binding data using the LIGAND program (34) indicated the data were fit by a one-site model ($K_D = 250 \pm 675$ pM, $p > 0.05$).

Affinity Labeling of High M_r Ligand-Receptor Complexes—Our earlier experiments (25) indicated that high M_r cross-linked ligand-receptor complexes were recovered at low concentration and that some form of enrichment would be necessary to study them. To accomplish this, receptor- 125 I-TNF complexes were formed on HeLa membranes in the presence and absence of excess unlabeled TNF, and the membranes were solubilized and fractionated by gel filtration. Peak fractions were pooled (Fig. 3), cross-linked with BSOCOES, and analyzed by SDS-PAGE (Fig. 3, inset). Using this protocol, a high M_r complex (310,000) and lower M_r bands (95,000 and 75,000) were affinity labeled (pool 1). These complexes were absent when competitor was included in the initial binding reaction (pool 2), indicating that the high M_r complexes were formed by specific binding of 125 I-TNF to receptors.

Binding of Cross-linked TNF and Analysis of TNF-Receptor Complexes by Reversal of Cross-linking—Earlier experiments had shown that TNF trimers could be stabilized against dissociation by introducing cross-links between TNF subunits (19). This approach was used for studying the association of cross-linked TNF and cell surface receptors. The 125 I-TNF was reacted with 1 mM DSS to introduce limited cross-linking such that SDS-PAGE resolved DSS- 125 I-TNF into monomers, dimers, and small amounts of trimers. Cross-linked 125 I-TNF was bound to HeLa cells with a K_D similar to that of untreated 125 I-TNF, since their binding was identically competed by unlabeled TNF (Fig. 4).

Covalent bonds with BSOCOES can be cleaved at pH 11.6 (32), whereas cross-links with DSS are resistant. These properties were used in combination with two-dimensional SDS-PAGE to examine the subunit structure of the receptor. For these experiments, complexes of DSS- 125 I-TNF with receptors were formed on HeLa cells at 4 °C. After cross-linking with BSOCOES, cell membranes were prepared and solubilized in 2% SDS and 50 mM DTT at 100 °C. An autoradiograph showing the separation by SDS-PAGE of these affinity labeled proteins is above the autoradiograph of the second dimension SDS-PAGE in Fig. 5. The first dimension gel separates free 125 I-DSS-TNF into monomers (M), dimers (D), and trimers (T) and separates the covalent products formed between TNF and the receptor. For the cross-link reversal

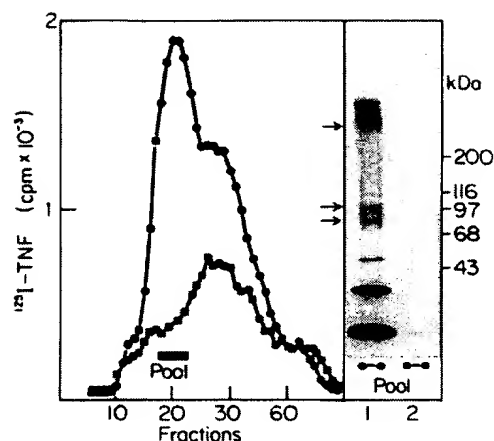


FIG. 3. Affinity labeling of high M_r TNF-receptor complexes on cell membranes. A, membranes from 1.3×10^6 HeLa cells were incubated in 4 ml for 5 h at 4 °C with 15 ng of 125 I-TNF in the presence and absence of 100-fold excess unlabeled TNF. The membranes were centrifuged to remove unbound TNF, and membrane pellets were solubilized with Triton X-100. Aliquots (0.1 ml) from total binding (●) and nonspecific binding (■) assays were applied to an Aca34 column (0.7 \times 28 cm) equilibrated in 0.1% Triton X-100, 5 mM $MgCl_2$, and 50 mM HEPES, pH 7.5. Fractions (0.2 ml) were collected, and 0.04-ml aliquots from corresponding fractions were counted. B, fractions 18–22 were pooled, cross-linked with 2 mM BSOCOES for 10 min at 4 °C and compared on an equal protein basis by SDS-PAGE (nonreducing) and autoradiography (inset); 1 shows the total binding pool and 2 the pool of nonspecific binding. Arrows, from top to bottom, indicate cross-linked complexes corresponding to $M_r = 310,000 \pm 5,000$ ($n = 5$), $95,000 \pm 1,000$ ($n = 9$), and $75,000 \pm 1,200$ ($n = 9$). The positions of the reference proteins myosin (200,000), β -galactosidase (116,250), phosphorylase B (97,400), bovine serum albumin (66,200), and ovalbumin (42,699) are indicated.

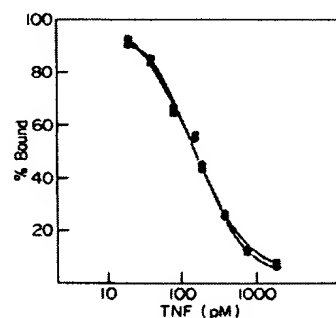


FIG. 4. Binding of DSS-cross-linked 125 I-TNF and 125 I-TNF to HeLa cells. 1 ng of each protein was incubated with 10^6 HeLa cells in 0.1 ml for 5 h at 4 °C in the presence or absence of increasing concentrations of unlabeled TNF. Mean total ($n = 3$) and nonspecific binding ($n = 2$) with 100-fold excess unlabeled TNF were 9729 and 2045 cpm for DSS- 125 I-TNF (■), 9478 and 1911 cpm for 125 I-TNF (●), respectively.

experiments, duplicate first dimension gels were incubated in reversal buffer (see "Materials and Methods"), re-equilibrated in sample buffer, and electrophoresed into the second dimension. Due to the partial reversal of cross-linking, where cross-linked TNF trimers are initially present in the first dimension gel, trimers, dimers, and monomers are resolved in the second dimension gel; and where dimers are initially present, dimers and monomers are found in the second dimension. The products derived from the 75,000 band (labeled B) are a M_r 75,000 spot on the diagonal and TNF monomer. This indicates that the M_r 75,000 band contains TNF monomer cross-linked to a putative receptor subunit of $M_r \sim 60,000$. Similarly, the M_r

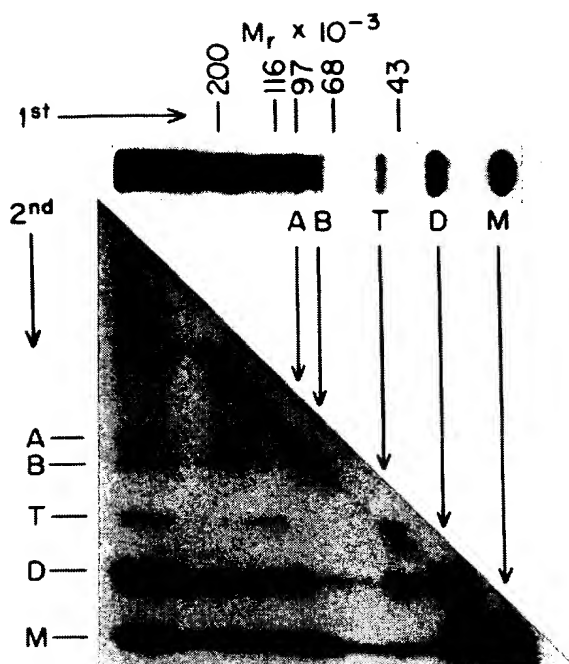


FIG. 5. Cross-linking reversal in two-dimensional SDS-PAGE. TNF-receptor complexes were formed by incubating 0.5 ng of DSS- ^{125}I -TNF/ 10^6 HeLa cells in 0.1 ml for 4 h at 4 °C. Bound TNF was cross-linked to the cells with 2 mM BSOCOES. Membranes were prepared and extracted at 100 °C in SDS-PAGE sample buffer with 50 mM DTT. Following electrophoresis in a 4–12% gradient gel, one lane from the first dimension gel was fixed and stained. The corresponding autoradiograph and reference marks are mounted horizontally above the second dimension autoradiograph. Before the second dimension electrophoresis, a duplicate lane from the first dimension gel was soaked in cross-link reversal buffer (see "Materials and Methods"). The positions of TNF monomers, dimers, and trimers are indicated by M, D, and T, respectively; and the 95,000 and 75,000 cross-linked complexes are indicated by A and B, respectively.

95,000 spot (labeled A) on the diagonal and TNF dimers (plus monomers) beneath it indicate that the M, 95,000 band contains receptor subunit plus cross-linked TNF dimer. The M, 95,000 band may also contain two TNF monomers independently cross-linked to the receptor polypeptide. TNF trimers (plus dimers and monomers) are derived from the first dimension gel near the M, 116,000 marker where the cross-linked moiety is not well resolved. Taken together, these results suggest that monomers, dimers, and trimers of DSS- ^{125}I -TNF (M, 17,350, 34,700, and 52,050) are initially cross-linked to a receptor subunit of M, ~ 60,000 forming, at least in part, the complex pattern of cross-linked proteins observed. The second dimension gel also shows that the 95,000 and 75,000 species (labeled A and B at the left margin in Fig. 5) are derived from the M, 150,000 to 175,000 region and from unresolved higher M, complexes. This suggests that the receptor component in the 75,000/95,000 complexes becomes cross-linked either to itself or to additional proteins.

Biotinylation of TNF and Characterization of ^{125}I -Biotinylated-TNF-Receptor Complexes—Adsorption of soluble TNF-receptor complexes based on the high affinity interaction between biotinylated TNF and streptavidin coupled to Sepharose-facilitated studies of the specificity of affinity labeling. Biotinylated and radioiodinated TNF (^{125}I -bio-TNF) was bound by HeLa cells in an identical manner as ^{125}I -TNF (Fig. 6A). The ^{125}I -bio-TNF retained biological activity, as it was fully active in cytotoxicity assays (Fig. 6B). Adsorption of this ^{125}I -bio-TNF by streptavidin-Sepharose beads resulted in a 9-

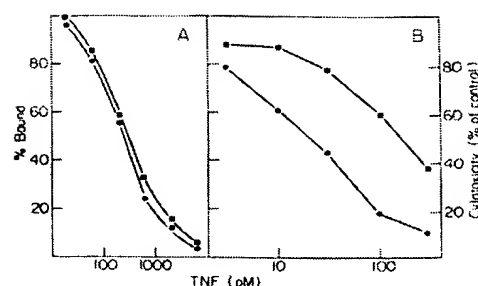


FIG. 6. Competitive binding and cytotoxicity of ^{125}I -biotinyl-TNF. A, 1 ng ^{125}I -TNF (●) or 1 ng ^{125}I -bio-TNF (■) was incubated in 0.1 ml with 10^6 HeLa cells/ml in the presence or absence of unlabeled TNF for 5 h at 4 °C. Specific binding for the biotinylated radioligand was 90% of the counts bound. B, ^{125}I -bio-TNF was adsorbed in a 1-h incubation with 50 μl of 1:1 suspension of streptavidin-Sepharose beads (■) or was mock adsorbed by Sepharose 6B beads alone (●) and then centrifuged to obtain supernatant fractions. Dilutions of the supernatants were tested by 18-h cytotoxicity assay on confluent Hela monolayers with 10 $\mu\text{g}/\text{ml}$ cycloheximide (25).

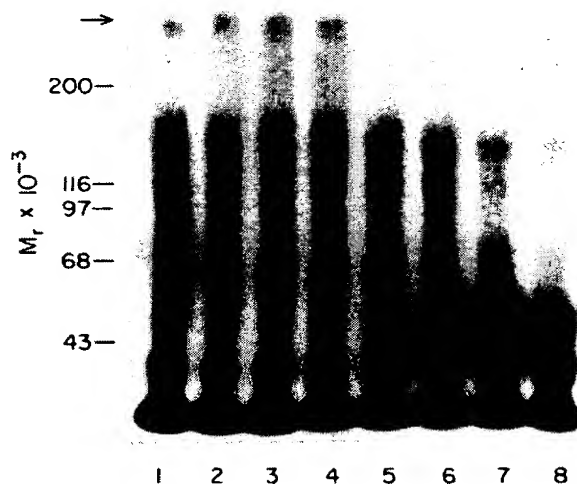


FIG. 7. Competitive inhibition of the M, 310,000 cross-linked complex. 2 ng of ^{125}I -bio-TNF were incubated in 0.14 ml for 18 h at 4 °C with Triton X-100 extract from HeLa cell membranes in the presence or absence of unlabeled TNF or other proteins. Each total incubate was cross-linked by the addition of BSOCOES to a final concentration of 2 mM. This reaction was quenched with 0.1 M glycine. 50- μl suspension of streptavidin-Sepharose beads was added to each total incubate. After 60 min at 4 °C, the incubates were diluted to 1.5 ml, beads were collected by centrifugation, and supernatants were discarded. The beads were resuspended in SDS-PAGE sample buffer without DTT and heated at 100 °C. Competitors added to the binding incubates were: 1 and 2, 100 ng of human recombinant interleukin-1 and interferon- γ ; 3, no competitor; 4–8, 1, 3, 10, 30, and 100 ng of TNF. The arrow shows the position of the M, = 310,000 cross-linked complex.

fold decrease in cytotoxic activity suggesting that ~90% of the ^{125}I -bio-TNF was bound to the streptavidin.

The ^{125}I -bio-TNF was incubated with Triton X-100 extracts of HeLa cell membranes in the presence and absence of interleukin-1, interferon- γ , and varying concentrations of TNF. The total binding mixtures were cross-linked with BSOCOES and concentrated by adsorption to streptavidin-Sepharose beads. SDS-PAGE under nonreducing conditions (Fig. 7) showed that interleukin-1 and interferon- γ (lanes 1 and 2) did not inhibit affinity labeling of 310,000 (arrow) and M, 150,000–175,000 bands. On the other hand, concentrations of TNF that competed with ^{125}I -TNF in binding assays (lanes 4–8), inhibited formation of these bands. Comparison of the

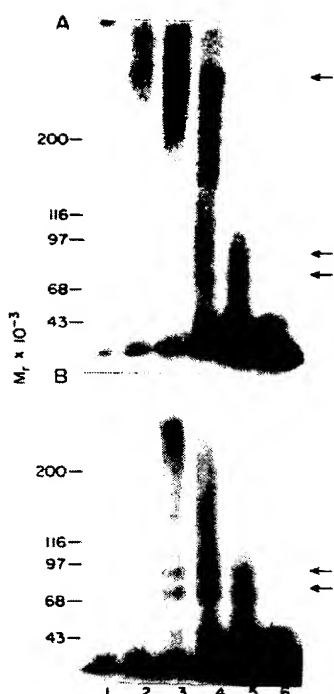


FIG. 8. Reduction of the M_r 310,000 complex with DTT. ^{125}I -bio-TNF was incubated with HeLa cell membranes, and the bound radioligand was cross-linked with 2 mM BSOCOES. The membranes were extracted at 100°C with 2% SDS and protease inhibitors (see "Materials and Methods"). After centrifugation at $150,000 \times g$, the supernatant was applied to a Sepharose 6B column (1.5×26 cm) equilibrated with 0.1% SDS and 50 mM Tris buffer, pH 6.8. Aliquots from the eluted fractions (lanes 1–6) were examined by SDS-PAGE unreduced (A) or after treatment with 50 mM DTT (B).

changes in intensity of these bands by densitometry (data not shown) revealed that each band was proportionately decreased by increasing concentrations of TNF. This result provided additional evidence for ligand-specific high affinity binding of TNF to such high M_r complexes.

The role of disulfide bonds in the formation of high M_r complexes was investigated by affinity labeling receptors on HeLa cell membranes. The bound ^{125}I -bio-TNF was cross-linked with BSOCOES, and the membranes were solubilized with 2% SDS at 100°C . These extracts were fractionated by gel filtration on a Sepharose 6B column. Analysis of the resulting column fractions by SDS-PAGE (Fig. 8A) showed a predominant M_r 310,000 band (lanes 2–4) when untreated with DTT. Inclusion of 100-fold excess unlabeled TNF in the binding incubation prevented the formation of this band, whereas inclusion of 10 mM biotin did not (results not shown). This indicated that formation of the complex was due to the specific binding of TNF and not due to binding of biotin. When these same fractions were treated with 50 mM DTT before electrophoresis (Fig. 8B), the M_r 310,000 band decreased and the M_r 95,000 and 75,000 bands were correspondingly increased. These results suggest that disulfide bonds have some role in stabilizing receptor-TNF complexes. Whether such bond(s) are interchain or intrachain is unclear due to the cross-linking introduced.

Transfer of Fractionated Receptors to Nylon Membranes and ^{125}I -TNF binding to Receptor Subunits—In order to study the role of disulfide bonds in the association of receptor subunits, HeLa membranes were solubilized with Triton X-100, fractionated by SDS-PAGE with or without reduction, and transferred to nylon membranes for ^{125}I -TNF binding

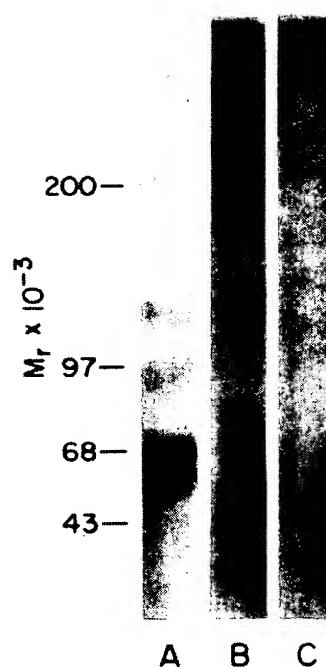


FIG. 9. Effect of reduction on ligand binding to TNF receptors on nylon transfer membranes. HeLa membranes were prepared and solubilized with Triton X-100 in the presence of protease inhibitors (see "Materials and Methods"), fractionated by 4–8% SDS-PAGE, and electrophoretically transferred. Strips of the solid support medium were incubated in 1% hemoglobin prior to ligand binding. A, incubation with ^{125}I -TNF alone. B, incubation with ^{125}I -TNF and 100-fold excess unlabeled TNF. C, extract treated with 25 mM DTT at 4°C for 3 min before electrophoresis and incubation with ^{125}I -TNF as in A. Positions of prestained M_r markers are indicated. The mean and standard error estimates for the M_r = 70,000 and 60,000 ^{125}I -TNF-binding proteins under nonreducing conditions of electrophoresis were $69,700 \pm 1,400$ and $58,000 \pm 1,700$ ($n = 5$).

assay. Such transfer experiments showed two ^{125}I -TNF binding proteins of M_r 70,000 and 60,000 (Fig. 9A). This binding was specific because it was completely inhibited by 100-fold excess of unlabeled TNF (Fig. 9B). TNF binding to these proteins was also abolished when the Triton X-100 extract was incubated with 25 mM DTT at 4°C before electrophoresis (Fig. 9C).

DISCUSSION

The structure of the TNF receptor was investigated by gel filtration, affinity labeling, and ligand blotting experiments. The results obtained suggest that TNF receptors on the cell surface consist of high M_r complexes containing at least two subunits. These putative subunits appear to be associated primarily by hydrophobic interactions rather than by covalent bonds because they are dissociated by SDS but not by Triton X-100. Furthermore, the binding site for TNF is stabilized by disulfide bonds because binding activity is lost in the presence of DTT.

TNF receptors, solubilized by Triton X-100 under conditions that maximize binding activity, elute in gel filtration as a major peak of binding activity at M_r 330,000 (Fig. 1). ^{125}I -TNF binding in this peak is inhibited by low concentrations of unlabeled TNF (Fig. 2) indicating high affinity binding. TNF-receptor complexes preformed on HeLa membranes and solubilized in Triton X-100 elute in gel filtration with M_r ~350,000 (25). Similarly formed complexes which were cross-linked after gel filtration (Fig. 3) exhibited mobility in SDS-PAGE corresponding to M_r = 310,000. These experiments

suggest that the same complexes were identified, but discrepancies in M_r estimates may be due to differences in their shape (35) or in the bound detergent in samples analyzed by gel filtration (36). Binding of ^{125}I -bio-TNF to the $M_r = 310,000$ complex is also competed by low concentrations of unlabeled TNF (Fig. 7). This result suggests the receptor complex itself is M_r 260,000–295,000, assuming that one TNF molecule is bound, and the mass of TNF monomer or trimer must be subtracted from the M_r 310,000 band.

Previous characterization of the TNF receptor using affinity labeling techniques identified TNF-receptor complexes of M_r 75,000 (21, 22, 29) and 92,000–100,000 (21, 22, 28, 29, 37) in diverse cell lines. In the present investigation, the relationship between the 310,000 M_r band and the M_r 95,000 and 75,000 bands in HeLa cells was investigated using a combination of irreversible and reversible cross-linking reagents and two-dimensional SDS-PAGE (Fig. 5). In these experiments, TNF itself is resolved into three bands corresponding in the greatest amount to TNF monomers, with less dimer and least trimer (Fig. 5). This complexity increases when TNF is cross-linked to receptors, since TNF has been shown to bind to receptors as a trimer (19) yet upon cross-linking to receptors, greater proportions of TNF monomers and dimers are observed. This quantitative inversion of cross-linked products may be explained by the requirement for three molecules of a bifunctional reagent to cross-link a trimer to one receptor protein compared to one molecule of reagent to cross-link one TNF monomer to a receptor protein. Thus, at limiting concentrations of bifunctional reagent, TNF is cross-linked to receptor in greater proportion by one cross-link than by multiple cross-links. TNF monomers are cross-linked to a M_r 75,000 component and dimers to a M_r 95,000 component. Subtracting the mass of TNF monomer from 75,000 and the mass of TNF dimer from 95,000 provides indirect evidence for a receptor subunit of $M_r \sim 60,000$. In addition, the M_r 75,000 and 95,000 bands appear to derive from a M_r 150,000 to 175,000 component suggesting that an additional protein mass is cross-linked. The predominant affinity labeling appears to involve direct cross-linking of TNF to a $M_r \sim 60,000$ receptor subunit. Secondary cross-linking may occur between this 60,000 protein, another receptor component and TNF forming the 150,000–175,000 band. Other interpretations of these data are possible; however, there is additional evidence for two receptor subunits.

The role of disulfide bonds in stabilizing the M_r 310,000 complex was investigated, since such bonds have been shown to stabilize other receptors containing multiple subunits (38). Treatment of M_r 310,000 cross-linked complexes with DTT resulted in the appearance of M_r 95,000 and 75,000 bands (Fig. 8). This finding suggests that disulfide bonds may stabilize the interaction of the putative M_r 60,000 subunit with the rest of the complex. Such a complex should resist dissociation by SDS. To examine this possibility, the solubilized receptor was treated with 1% SDS, fractionated by SDS-PAGE, and transferred to solid support media. Under these conditions, the TNF binding activity was present in two discrete bands of M_r 60,000 and 70,000 (Fig. 9). In repeated experiments, no binding activity was detected at higher M_r . Furthermore, when the Triton X-100 extracts were incubated with DTT, the TNF binding activity at M_r 60,000 and 70,000 was lost. These results suggest that interchain disulfide bonds, if present, have only a minor role in stabilizing high M_r receptor complexes. On the other hand, intrachain disulfide bonds have apparently an important role in maintaining TNF binding (Fig. 9C), since this activity is lost in the presence of DTT. In a previous report, TNF binding activity from U-937

cells was found to elute during gel filtration over a broad range of molecular sizes with a peak centered at $M_r \sim 65,000$ (37). This peak may correspond to one of the shoulders observed in the elution profile of TNF receptors from HeLa cells ($M_r \sim 90,000$, Fig. 1). These results are consistent with a multimeric structure for TNF receptors stabilized primarily by hydrophobic interactions.

Indirect evidence from affinity labeling studies suggests that the TNF receptor contains associated subunits. Direct evidence for two TNF binding proteins of $M_r = 60,000$ and 70,000 is provided by ligand blotting experiments. It is also apparent from gel filtration of Triton X-100 extracts and from cross-linking studies with both intact cells and membranes that these subunits associate to form larger complexes. Such complexes may be formed by multiple 60,000 and 70,000 components. The relationship between these proteins is unknown. It is possible that the 60,000 component is a proteolytic fragment despite the use of protease inhibitors. Alternatively, the 70,000 component may be a glycosylated form of the same polypeptide. Concanavalin A has been reported to modulate TNF binding activity, suggesting that the TNF receptor is glycosylated (39). In addition, cross-linked TNF-receptor complexes were reported to bind to concanavalin A (26). It is also possible that another as yet unidentified polypeptide is part of the TNF receptor. A band of $M_r = 138,000$ has been detected by affinity labeling experiments in MCF-7 cells sensitive to the cytotoxic activity of TNF, whereas variants of these cells resistant to TNF do not contain such a band (30). A band of this M_r may arise as a cross-link product of two receptor subunits and TNF, suggesting a possible functional correlation for the assembly of these receptor complexes. Receptor dimerization enhances affinity and signal transduction in epidermal growth factor receptors (40). Similarly, the affinity for TNF- α or TNF- β (39) and signal transduction may be modulated by the multimeric structure of the TNF receptor.

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immunodominant region of the autoantigen MBP. This may provide insight into the molecular mechanisms of MS and help in the design of new specific therapeutic approaches.

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- Myelin basic protein-specific T cell lines were grown from peripheral blood mononuclear cells at 200,000 cells per well in the presence of human MBP (10 µg/ml). Under these conditions 1 to 20% of the wells were positive for MBP; therefore, most lines are likely to have been generated from a single MBP-reactive T cell. Cells were stimulated two times with MBP and tested for their peptide specificity by use of a panel of 13 overlapping synthetic MBP peptides. All cell lines analyzed reacted specifically with one of the 13 synthetic MBP peptides (4). After a third stimulation with the specific MBP peptide, RNA was extracted from cell culture pellets (20,000 to 50,000 cells) by extraction with guanidinium isothiocyanate/phenol chloroform and isopropanol precipitation in the presence of carrier tRNA. Single-stranded cDNAs were synthesized with oligo-dT and avian myeloblastosis virus reverse transcriptase. PCR amplification was done with a panel of 19 oligonucleotides corresponding to the CDR2 region of the TCR β chain and a C_β primer. Amplifications were done for 30 cycles (94°C, 1 min; 55°C, 2 min; and 72°C, 3 min) with 1 µg of each primer in 50-µl reactions. Amplified products were separated in 1% agarose gels, transferred to nitrocellulose, and hybridized with an internal oligonucleotide probe. Probes were end-labeled with [γ -³²P]ATP (adenosine triphosphate) and T4 polynucleotide kinase to a specific activity of 10⁸ cpm/µg and hybridized. Blots were washed at a final stringency of 6× SSC (saline sodium citrate) at 70°C and autoradiographed for 2 to 18 hours. T cell lines that were positive for more than two V_β segments were considered not to be derived from a single MBP-reactive T cell and were therefore excluded from analysis. For sequencing, amplification was performed with a V_β17 primer specific for the leader segment, which contained an internal Pst I restriction site. Amplified DNA was treated with proteinase K, extracted with phenol chloroform, precipitated with ethanol, and digested with restriction endonucleases Bgl II and Pst I. Gel-purified DNA was ligated into M13mp19, and single-stranded DNA was sequenced by the dideoxy method. Negative controls were included during the procedure to test for possible contamination of RNA samples or reagents used for cDNA synthesis and amplification.
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A Receptor for Tumor Necrosis Factor Defines an Unusual Family of Cellular and Viral Proteins

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Tumor necrosis factor α and β (TNF- α and TNF- β) bind surface receptors on a variety of cell types to mediate a wide range of immunological responses, inflammatory reactions, and anti-tumor effects. A cDNA clone encoding an integral membrane protein of 461 amino acids was isolated from a human lung fibroblast library by direct expression screening with radiolabeled TNF- α . The encoded receptor was also able to bind TNF- β . The predicted cysteine-rich extracellular domain has extensive sequence similarity with five proteins, including nerve growth factor receptor and a transcriptionally active open reading frame from Shope fibroma virus, and thus defines a family of receptors.

TUMOR NECROSIS FACTOR α (TNF- α , cachectin) and β (TNF- β , lymphotoxin) are structurally and functionally homologous proteins secreted by activated macrophages and lymphocytes, respectively (1). These cytokines have pleiotropic activities in vitro and in vivo, including cytotoxic effects against tumors and virus-infected cells, stimulation of interleukin-1 secretion, stimulation of prostaglandin E2 and collagen production, inhibition of lipogenic gene expression in adipocytes, and stimulation of various immune effector cells (2). Clinical interest has focused on TNF because it appears to be a common

mediator of inflammation, endotoxin-induced shock (1), and the wasting syndrome commonly observed in chronic infections and neoplastic disease (3). TNF receptors appear on virtually all somatic cells (1), and generally the ligands cross-compete for binding (4), suggesting they share a common receptor. As an aid to studying the TNF system in molecular detail, we isolated a cDNA clone of the receptor.

The SV40-transformed human lung fibroblast cell line WI26-VA4 was used as a source of mRNA for construction of a cDNA library. This cell line binds both TNF- α and - β and displays multiple affinity classes; approximately 23,000 binding sites per cell (N) were detected with ¹²⁵I-TNF- α that could be fit to two affinity classes, low ($K_{d1} = 0.16 \pm 0.10$ nM⁻¹, $N_1 = 19,700 \pm$

Immunex Corporation, Seattle, WA 98101.

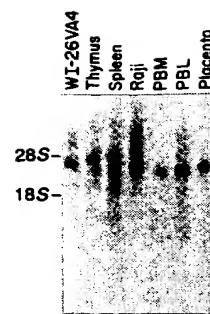
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4,800) and high ($K_{a2} = 6.2 \pm 3.9 \text{ nM}^{-1}$, $N_2 = 3,000 \pm 1,400$) (Fig. 1A). TNF- β binds with lower affinity than TNF- α and the ligands cross-compete for binding (Fig. 1B). Double-stranded cDNA was synthesized by standard procedures, inserted into the mammalian expression vector pDC302 (5) and a TNF receptor clone isolated by a direct expression approach. Plasmid DNA from about 1000 *Escherichia coli* (DH5 α) transformants were pooled, transfected into COS cells, and screened by contact autoradiography (6), which detects positive pools by the ability of those COS cells expressing TNF receptor inserts to bind ^{125}I -labeled TNF- α . After screening 175,000 clones, one positive pool (#737) was obtained, subdivided, and converged to a single clone in two cycles of this procedure. By autoradiographic plate binding (6), the pure clone when transfected into COS cells expressed a receptor that bound both ^{125}I -TNF- α and - β ; binding of either ligand was completely inhibited by a 200-fold excess of the same or homologous unlabeled cytokine (7). Quantitative *in situ* binding studies of the COS-expressed receptor with ^{125}I -TNF- α agreed with these results and showed the binding to be complex (Fig. 1C). As with the native

WI26-VA4 receptor, the recombinant COS receptor displayed both low ($K_{a1} = 0.18 \pm 0.06 \text{ nM}^{-1}$) and high ($K_{a2} = 10.1 \pm 1.0 \text{ nM}^{-1}$) affinity classes for ^{125}I -TNF- α . TNF- β bound with lower affinity and competitively inhibited ^{125}I -TNF- α binding (Fig. 1D). Thus, ligand binding properties of both the native and recombinant receptor appear similar. The origin of the multiple affinity classes for TNF- α is unclear. Indeed, most workers (1, 4, 8, 9), but not all (10), have reported monophasic Scatchard plots for TNF- α . However, TNF- α is predominantly a homotrimer (11) and therefore intrinsically capable of multivalent binding. In one report (12), differential biological effects could be related to biphasic binding of TNF- α . While not necessarily sharing a common origin, multiple affinity classes are a common feature of many receptor systems (13).

The isolated TNF receptor cDNA was used as a probe to analyze the mRNA expressed in a variety of cell lines and tissues (Fig. 2). A single size class of transcripts of ~4.5 kb was detected in WI26-VA4, Raji cells (a B lymphoblastoid line), LPS-stimulated peripheral blood monocytes (PBM), induced peripheral blood T cells (PBL), and

Fig. 2. RNA blot analysis of TNF receptor mRNA. Polyadenylated RNA (3.5 μg) was used from each source, except placental tissue (5 μg total RNA). PBL were cultured for 6 days in IL-2 and OKT3 monoclonal antibody, then restimulated for 8 hours with concanavalin A (Con A) and PMA (6). RNA was fractionated on a 1.1% agarose-formaldehyde gel, blotted onto Hybond-N (Amersham), and hybridized with a labeled antisense RNA probe prepared from the 630-bp Not I-Bgl II fragment of the TNF receptor cDNA that had been subcloned into a Bluescript plasmid (Stratagene). Filter hybridization and washing conditions were as described (5). Variable exposure times were used in preparing the figure.



placental tissue. A transcript of slightly larger size (~5.0 kb) was detected in thymic tissue, and splenic tissue contained transcripts of both size classes. The origin of these differences is not clear, but the presence of TNF receptor transcripts in these different cells is consistent with the near ubiquitous distribution of the receptor.

The 3.7-kb insert of clone 737 was subcloned and sequenced (5) (Fig. 3). The cDNA contains a string of adenines at the 3' end and an upstream consensus polyadenylation signal. The discrepancy between the size of the isolated cDNA and that of the transcripts estimated from Northern analysis may be due to a deficiency of 5' sequences in this clone. It is also possible that alternative polyadenylation signals are utilized. Upstream of the polyadenylation site is a 299-bp segment that has homology to the Alu family of repetitive sequences (14). The sequence contains a single large open reading frame encoding 461 amino acids with features typical of an integral membrane protein (15). The initiating methionine precedes 22 hydrophobic residues characteristic of a leader sequence; the most probable cleavage site (16) predicts Leu²³ as the mature NH₂-terminus. Another hydrophobic region of 30 amino acids is located between residues 258 and 287, bordered by charged residues at either end (Asp²⁵⁷ and Lys²⁸⁸⁻²⁹⁰), consistent with a transmembrane segment that makes a single helical span. Immediately upstream of this element is a region of 57 amino acids rich in threonine, serine, and proline residues. Such a composition is indicative of O-linked glycosylation sites containing sialic acid and is found in similar extracellular regions of several receptors, including those for nerve growth factor (NGF) (17) and low density lipoprotein (LDL) (18). The NH₂-terminal 162 amino acids (positions 39 to 200) are rich in

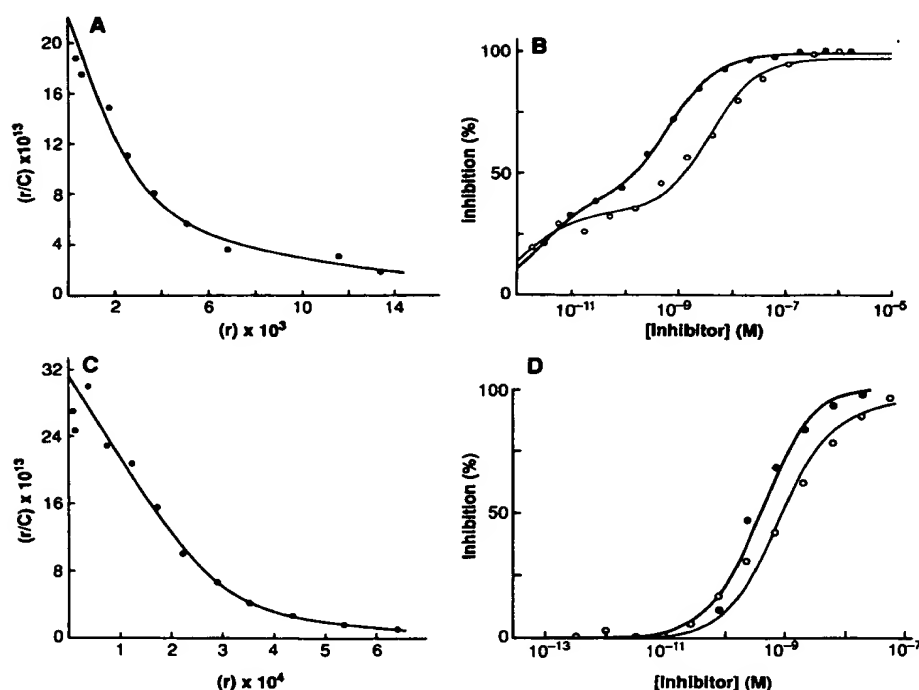


Fig. 1. TNF binding characteristics of native and recombinant TNF receptors (31). (A) Direct binding of ^{125}I -TNF- α to WI26-VA4 cells (Scatchard coordinate system). (B) Inhibition of ^{125}I -TNF- α binding to WI26-VA4 cells by unlabeled TNF- α (●) and TNF- β (○). TNF- α inhibition: $K_{1,1}$ (low affinity) = $1.6 \pm 0.2 \text{ nM}$; $K_{1,2}$ (high affinity) = $0.8 \pm 0.1 \text{ pM}$. TNF- β inhibition: $K_{1,1}$ (low affinity) = $0.29 \pm 0.06 \text{ nM}$; $K_{1,2}$ (high affinity) = $1.3 \pm 0.6 \text{ pM}$. (C) Direct binding of ^{125}I -TNF- α to recombinant (COS) TNF receptor. (D) High affinity site inhibition of ^{125}I -TNF- α binding to recombinant (COS) TNF receptor by unlabeled TNF- α (●) or - β (○). K_1 (α) = $6.7 \pm 2.9 \text{ nM}$; K_1 (β) = $3.3 \pm 0.8 \text{ nM}$. C, free concentration of TNF (molar); r, molecules of TNF bound per cell. All parameter values are \pm standard error. Data fit to one or two site models as described (32).

cysteines (22 residues) and also contain two potential N-linked glycosylation sites. The receptor terminates in a cytoplasmic domain of 174 amino acids, rich in serines (18%), six of which are contiguous. Five cysteines and one potential N-linked glycosylation site are also present in this domain.

A computer search of several sequence databases (19) queried with the entire 439-residue sequence of the mature TNF receptor revealed five proteins with striking similarity: human and rat NGF receptor, CD40,

cDNA clone 4-1BB, and T2 (Fig. 4). Four of these are transmembrane proteins, two of which are known receptors (for human and rat NGF). CD40 is a B cell-localized surface antigen, found also on neoplastic cells of epithelial origin, that becomes phosphorylated in the cytoplasmic domain after binding the CD40-specific monoclonal antibody G28-5 (20). Clone 4-1BB was identified as a murine cDNA from induced helper and cytolytic T cell clones (21). Both molecules have been suggested to be cytokine recep-

tors for unidentified ligands. All identity between these four proteins is localized to the cysteine-rich regions of the extracellular domains; no homology was detected between the TNF receptor cytoplasmic domain and any proteins in the database. T2 is a transcriptionally active open reading frame from the Shope fibroma virus (SFV), a poxvirus that produces invasive malignancies in newborn rabbits (22). Although dominated by 22 conserved cysteines, the alignment is also reinforced by other conserved amino acids, particularly tyrosine, glycine, and proline. Thus, the extracellular domains of these molecules, presumably heavily disulfide bonded, probably share a common structural motif. Central to this motif would appear to be repeating homologous domains. Several groups have shown that the cysteine-rich regions of NGF receptor and CD40 can be resolved into either pseudo twofold repeats of about 80 amino acids or pseudo fourfold repeats of about 40 residues (17, 20). Similar repeats can be shown with the TNF receptor and T2, consistent with all these genes having arisen by duplication and divergence from a common gene. Since both NGF and TNF are oligomeric, repeating substructures in their receptors may aid in binding and predicts that the putative ligands for CD40 and 4-1BB may also be oligomers. The net charge associated with the cysteine-rich domains of these family members varies (-19 for NGF receptor; +1 for TNF receptor), which may be related to ligand specificity. Presumably, it is this NH₂-terminal region that contains the TNF binding site. Multiple lines of evidence have localized the (apoprotein B) ligand binding site of the LDL receptor to the NH₂-terminal (60-kD), cysteine-rich

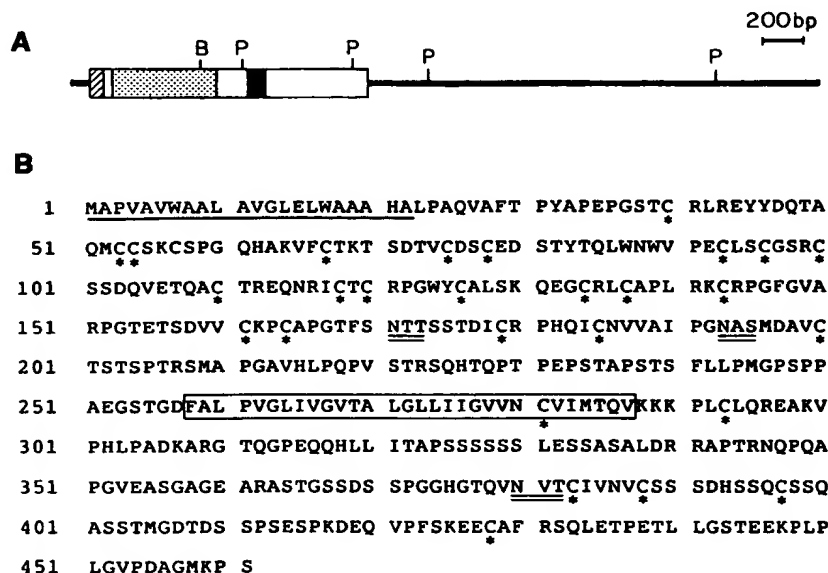
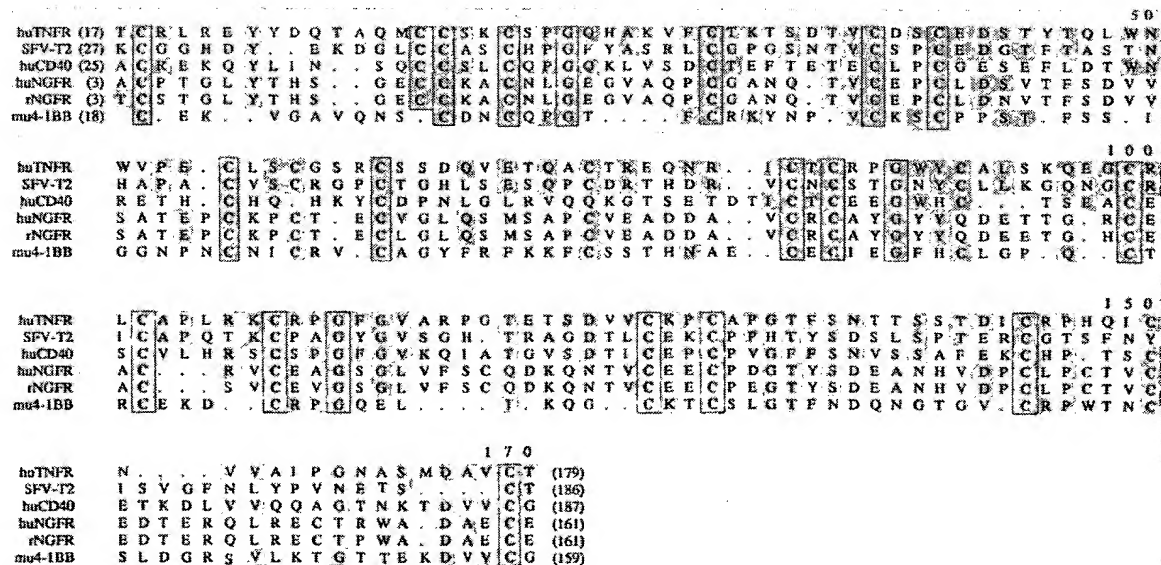


Fig. 3. Sequence of the human TNF receptor cDNA clone. (A) Schematic representation and restriction map of the cDNA. The entire coding region is boxed. The leader is hatched, the cysteine-rich region is shown stippled, and the transmembrane segment is solid. B = Bgl II; P = Pvu II. (B) The deduced amino acid sequence of cDNA coding region. The leader region is singly underlined, the transmembrane domain is shown boxed, potential N-linked glycosylation sites are doubly underlined, and cysteines are identified by an asterisk. The entire nucleotide sequence is available upon request and has been deposited at GenBank, accession number M32315.

Fig. 4. Sequence similarities among the TNF receptor superfamily. Consensus alignment of residues from the cysteine-rich regions of human TNF receptor (huTNFR), T2 open reading frame of Shope fibroma virus (SFV-T2), human CD40 (huCD40), human and rat nerve growth factor receptor (huNGFR and rNGFR), and murine cDNA clone 4-1BB (mu4-1BB). Numbers at NH₂- and COOH-termini refer to residues as cited in publications describing cDNA cloning (17, 20, 21, 22); numbers at top right of each block mark residues from NH₂-terminus at top left. Shaded residues reflect those common to huTNF receptor and at least one other protein. Cysteines are in bold, and boxed residues are invariant.



domain (18).

Sequences containing cysteine-rich repeats are present in a number of proteins, including the CD18 adhesion molecules (23), epidermal growth factor (EGF) precursor, *Drosophila notch* protein, the *neu* oncogene, and the external domains of receptors for LDL, EGF, and insulin (18, 24). Although many of these proteins show homology to each other, we detect little similarity to the TNF receptor. Optimal alignments of family members using the National Biomedical Research Foundation (NBRF) ALIGN program (19) show the strongest similarity is between the TNF receptor and T2, with a score of 19 standard deviations (SD) above the mean score for an ensemble of randomly permuted molecules of the same lengths and amino acid composition. ALIGN scores greater than 3.0 are considered significant and indicate common ancestry. Almost 40% of the residues are identical, approaching the conservation level between many murine and human cytokines and their receptors (25). Slight variants of T2 may also exist in other poxvirus family members, and some of these viruses are strongly immunosuppressive (22). Although T2 possesses a signal peptide sequence, the molecule appears to lack a hydrophobic segment typical of transmembrane regions, suggesting that T2 may be a soluble entity secreted from virally infected cells. Thus, perhaps T2 may bind TNF, or another cytokine, serving to locally dampen the host immune response. The protective effects of such a "soluble receptor" would no doubt confer a selective advantage to the pathogen. CD40, however, is also similar to this TNF receptor (38.5% amino acid identity; 15.2 SD), yet does not bind TNF- α when expressed in COS cells at high levels in an immunoreactive form (26). TNF receptor is more distantly related to 4-1BB and NGF receptor (9.0 and 12.3 SD, respectively).

The signal transduction mechanism of TNF is unclear. The receptor cytoplasmic domain, as with other family members, shows no similarity with known proteins, including the cytoplasmic domain of the human T cell interleukin-1 (IL-1) receptor (6), despite the fact that TNF and IL-1 mediate many common biological activities (1). The TNF receptor expressed in COS cells does not bind radiolabeled human IL-1 α or - β , nor does the recombinant human IL-1 receptor bind TNF (7). No sequences present are typical of tyrosine kinases, protein kinase C, or phosphorylation sites corresponding to substrates for these kinases (27). The cytolytic activity of TNF, however, appears to depend on the presence of a 200-kD protein distinct from the receptor, and with which it comodulates (28).

Several groups have characterized TNF binding proteins from urine. Uromodulin is a renal glycoprotein that binds IL-1, IL-2, and TNF- α with high affinity, but does not inhibit ligand binding to their respective receptors and shows no sequence similarity to the TNF receptor reported here (29). Two groups have recently reported purification and sequencing of soluble TNF- α binding proteins from urine with molecular weights of 27 to 30 kD (30). However, the NH₂-terminal sequence of these proteins is not found in the predicted sequence of clone 737. TNF- α receptors on myeloid cells are probably different from those on cells of epithelial origin (8). An 80-kD form of the receptor contains O- and N-linked carbohydrate; a 60-kD form lacks O-linked carbohydrate, possesses a different form of N-linked carbohydrate, and displays different tryptic peptide maps. Monoclonal antibodies to these two receptors also do not cross-react. The receptor we have described may correspond to the 80-kD form. Affinity cross-linking of the recombinant receptor using either ¹²⁵I-TNF- α or - β shows a single species of 80 kD (7). Because the calculated protein is 46 kD, carbohydrate appears to be attached, and both O- and N-linked glycosylation sites are present in the sequence.

The availability of a full-length cDNA clone for a human TNF receptor will now permit detailed studies into the molecular mechanisms by which ligand-receptor interactions produce the pleiotropic effects of this important cytokine. Soluble, recombinant forms of this receptor may also be produced to explore the clinical value of TNF inhibition in pathological settings.

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31. COS cells were transfected with the vector pDC302 containing the TNF receptor cDNA insert (clone 737) or control vector lacking insert as described (5, 6). For quantitative in situ binding studies, transfected COS cells were replated (24 hours after transfection) into six well trays (CoStar) and analyzed 48 hours later at near confluence (6×10^5 cells per well). COS monolayers were washed once with phosphate-buffered saline (PBS), then incubated with ^{125}I -TNF- α at various concentrations in binding media [RPMI 1640, bovine serum albumen (10%), NaN_3 (0.1%), 20 mM Hepes, pH 7.4] at 4°C for 2 hours. Free ^{125}I -TNF- α was determined by counting gamma emissions in the supernatant. Monolayers were then washed once with ice-cold RPMI, detached with 0.1% trypsin in PBS, and counted to determine bound ligand. Nonspecific ligand binding was determined by inclusion of a 200-fold molar excess of unlabeled ligand. Inhibition assays used ^{125}I -TNF- α at 0.2 nM. Data were analyzed and theoretical curves plotted as described (6, 32). TNF- α and TNF- β (R&D Sciences) were radiolabeled using Iodogen (Pierce) to a specific activity of 2×10^{15} cpm/nmol (4). Radiolabeled TNF- α gel filtered as a single peak with an apparent molecular weight of 55 kD (7), consistent with a trimeric status (11).
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"The good news is we have the human genome. The bad news is the computer alphabetized it."

Highly efficient neutralization of HIV with recombinant CD4-immunoglobulin molecules

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THE human immunodeficiency virus type 1 (HIV-1) exploits the cell surface CD4 molecule to initiate the infection¹⁻⁴ which can lead, eventually, to acquired immunodeficiency syndrome (AIDS). The HIV-1 envelope protein, gp120, interacts specifically with CD4 and soluble CD4 molecules have been shown to inhibit HIV infectivity *in vitro*⁵⁻⁹. Effective inhibition *in vivo* may, however, require more potent reagents. We describe here the generation of molecules which combine the specificity of CD4 and the effector functions of different immunoglobulin subclasses. Replacing the VH and CH1 domains of either mouse γ 2a or μ heavy chains with the first two N-terminal domains of CD4 results in molecules that are secreted in the absence of any immunoglobulin light chains. We find that the pentameric CD4-IgM chimaera is at least 1,000-fold more active than its dimeric CD4-IgG counterpart in syncytium inhibition assays and that effector functions, such as the binding of Fc receptors and the first component of the complement cascade (C1q), are retained. Similar chimaeric molecules, combining CD4 with human IgG were recently described by Capon *et al.*¹⁰, but these included the CH1 domain and did not bind C1q. Deletion of the CH1 domain may allow the association and secretion of heavy chains in the absence of light chains¹¹, and we suggest that the basic design of our constructs may be generally and usefully applied.

We have previously produced chimaeric proteins consisting of different domains of CD4 and immunoglobulin constant region of mouse κ (M κ). If we denote the four suggested extracellular domains of CD4 protein starting at N-terminus by 1, 2, 3 and 4 (ref. 12), the following proteins were generated: 1·C κ , 1·2·C κ , 1·2·3·C κ and 1·2·3·4·C κ (ref. 13). All these

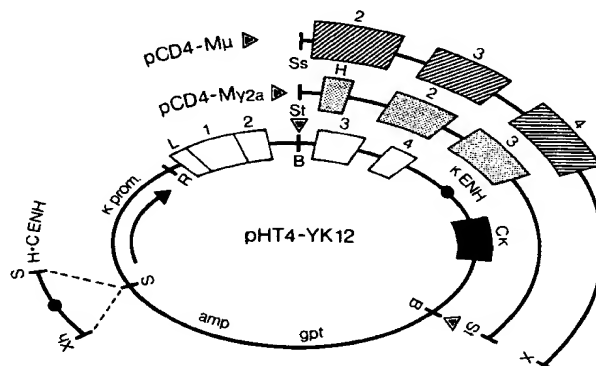
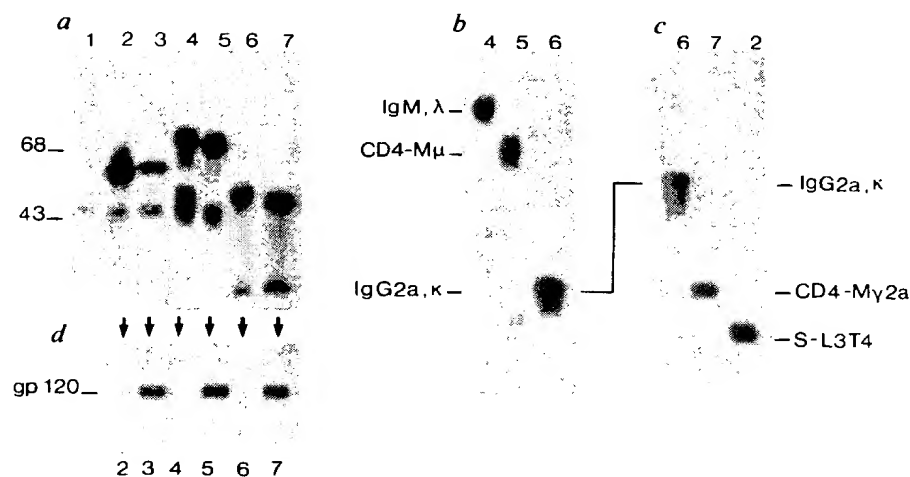


FIG. 1 Immunoglobulin-CD4 chimaeric constructs. The plasmid pHT4.YK12 encodes all four extracellular domains of CD4 linked to the mouse Ig κ constant regions. CD4-M μ and pCD4-M γ 2a encode the first two N-terminal CD4 domains linked to mouse Ig μ (CH2, CH3, CH4) and mouse Ig γ 2a (Hinge, C μ 2, C μ 3) constant regions, respectively. Numbers above the boxes refer to exons coding for different domains. ENH=Enhancer, L=Leader, H=Hinge, B=Bam HI, R=Eco RI, S=Sal I, Ss=Sst, St=Stu I, X=Xba and Xh=Xho I. METHODS. The CD4- κ expression vector pHT4-Y1 (ref. 9) was modified by replacing the immunoglobulin heavy chain promoter by a more efficient immunoglobulin κ promoter derived from the vector pKm 1 (ref. 32). The κ promoter was first subcloned into an intermediate vector PUC 18 as a Bgl II/Sal I fragment, then transferred as a 2.2 kilobase (kb) fragment [Hind III(blunt)/Eco RI] into pHT4-Y1 [Xba I(blunt)/Eco RI], to generate pHT4-YK12. The Bam HI fragment of pHT4-YK12 (encoding domains 3 and 4 of CD4 and C κ) was then replaced by either a 3.5 kb Sdt/Xba I fragment containing C μ 2, C μ 3 and C μ 4 constant region exons of mouse Ig μ or a 3.0 kb Stu I fragment encoding the hinge, C μ 2 and C μ 3 constant region exons of mouse Ig γ 2a. All fragments were blunt-ended before ligation into the vector. The constructs pCD4-M μ and pCD4-M γ 2a were completed by inserting a heavy chain enhancer as a Sal I/Xho I fragment into the unique Sal I site 5' of the promoter.

molecules retained their gp120 binding activity, suggesting that the first V κ -like domain is sufficient. We first attempted to produce secreted CD4-immunoglobulin chimaeras with specificity against gp120 by generating double transfectants producing 1·C κ and normal immunoglobulin heavy chains. This approach failed, however, and we could not detect assembly into complete molecules, even though a number of heavy chains with different variable regions were tested.

FIG. 2 Characterization of the chimaeric molecules. Stable transformants were obtained by transfecting CD4 constructs into X63-O myeloma cells by protoplast fusion. a-c, Western blot analysis of immunoprecipitated chimaeric or natural immunoglobulin molecules from cell culture supernatants after 10% PAGE run under reducing conditions (a) or after 3-7% gradient PAGE (b) or 7% PAGE (c) run under non-reducing conditions. Non-transfected X63-O (1), soluble murine CD4, L3T4 (ref. 9) (2), HT4-YK12 (3), B1.8 (IgM, λ) (4), CD4-M μ (5), UPC10 (IgG2a, κ) (6), CD4-M γ 2a (7). Samples 1-3 were immunoprecipitated with rabbit anti-mouse κ , samples 4 and 5 with rabbit anti-mouse IgM, samples 6 and 7 with protein A. All antibodies and protein A were immobilized on Sepharose 4B. The results were visualized with a mixture of rabbit anti-mouse Ig κ , μ and γ antibodies in a first step, followed by radioactive donkey-anti-rabbit immunoglobulin. Standard molecular weight markers are shown on the left (in thousands). The background bands in a (1-5), of relative molecular mass ~45,000 are due to rabbit IgG released from the Sepharose when the samples were prepared for electrophoresis. d, Co-immunoprecipitation under identical conditions as those in a-c, but in the presence of metabolically labelled gp120 from the supernatant of HIV-1-infected H9 cells, was followed by PAGE and fluorography. METHODS. Four million H9/HTLV-III cells³³ were pulse-labelled as previously



described³⁴ with [³⁵S]cysteine for 4 h. The cells were then transferred into 4 ml of fresh medium and incubated for a further 14 h. After the chase the cells were quickly removed by pelleting at 400g and the supernatant was subjected to 150,000g sedimentation for 1 h. Cleared supernatant (50 μ l) was then incubated with the indicated culture fluids for 14 h at 4 °C and the immunoprecipitations were performed as described above.

The successful approach relied on two observations. First, $1 \cdot 2 \cdot C\kappa$ and $1 \cdot 2 \cdot 3 \cdot 4 \cdot C\kappa$ were secreted, whereas $1 \cdot C\kappa$ and $1 \cdot 2 \cdot 3 \cdot C\kappa$ were not, suggesting to us that CD4 domains associate pairwise into two stable units consisting of $1 \cdot 2$ and $3 \cdot 4$ domains respectively. Second, the analysis of human heavy-chain disease proteins has shown that heavy chains of different subclasses can be secreted without light chains when the first constant region domain is deleted¹¹. We therefore constructed plasmids in which the exons encoding the first two N-terminal domains of CD4 were linked to all but the V_H1 and C_H1 domains of either the mouse μ heavy chain (CD4-M μ) or the mouse $\gamma 2a$ heavy chain (CD4-M $\gamma 2a$) (see Fig. 1).

Both CD4-M μ and CD4-M $\gamma 2a$ were found in the culture supernatants at levels of $1\text{--}5 \mu\text{g ml}^{-1}$ after the corresponding constructs were introduced into a myeloma cell line X63-0 (Fig. 2). Immunoprecipitations of the secreted proteins with relevant antibodies or protein A and subsequent western blot analyses showed that the molecules produced had the expected apparent molecular weights (Fig. 2). A similar analysis under non-reducing conditions indicated that CD4-M μ was most probably secreted as a pentamer, whereas CD4-M $\gamma 2a$ formed dimers, consistent with the fact that CD4-M $\gamma 2a$ bound to protein A (ref. 14) (Fig. 2b, c). Both CD4-M μ and CD4-M $\gamma 2a$, as well as the previously produced CD4-M κ , were able to bind to HIV gp120 (Fig. 2d). Because recombinant gp120 was not available, we used metabolically labelled preparations of gp120 derived from the supernatants of HIV-infected (H9) cell cultures in our co-immunoprecipitation tests.

Importantly, the effector functions of normal immunoglobulin molecules, such as binding to Fc γ receptors and C1q were kept intact in the hybrid molecules (Fig. 3). This suggests that removal of the CH1 domain does not create major structural alterations

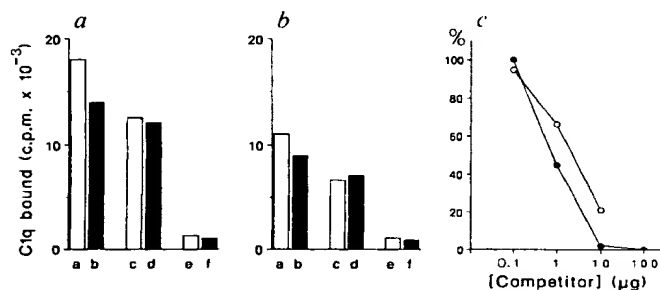


FIG. 3 Characterization of binding properties of CD4-immunoglobulin molecules. *a*, *b*, Clq binding assay. Microtitration plates were coated with purified CD4-immunoglobulin and natural control proteins of appropriate subclasses at the concentrations of $10 \mu\text{g ml}^{-1}$ (*a*) and $1 \mu\text{g ml}^{-1}$ (*b*). The direct binding of ^{125}I -labelled Clq was then measured. *a*, CD4-M $\gamma 2a$; *b*, OKT3 (IgG2a, K); *c*, CD4-M μ ; *d*, TEPC 183 (IgM, K); *e*, CD4-M κ ; *f*, BSA. *c*, Binding of CD4-immunoglobulin molecules to Fc γ receptors on the mouse macrophage cell line M29. Competition between ^{125}I -labelled CD4-M $\gamma 2a$ and the indicated amounts of CD4-M $\gamma 2a$ (○) and OKT3, (IgG2a) (●) proteins. METHODS. *a* and *b*, Polyvinyl chloride microtitration plates were coated with $100 \mu\text{l}$ of test and control proteins in saline at concentrations of $10 \mu\text{g ml}^{-1}$ (*a*) and $1 \mu\text{g ml}^{-1}$ (*b*). After overnight incubation the plates were blocked with 1% BSA solution and binding of ^{125}I -labelled human Clq ($\sim 2 \text{ ng}$, 50,000 c.p.m.) was measured after incubation for 6 h at room temperature. Radiolabelling of Clq (a gift from Dr A. Erdei) was carried out by the iodogen method, according to the recommendations of the manufacturer (PIERCE). *c*, Fc γ receptor binding was assayed by incubating 2×10^5 M29 mouse macrophage cells (a gift from Dr G. Stockinger) with ^{125}I -labelled CD4-M $\gamma 2a$ (200 ng), plus competitor proteins in $100 \mu\text{l}$ medium containing 5% fetal calf serum for 1 h at 4°C . After incubation the samples were centrifuged through a $200 \mu\text{l}$ cushion of fetal calf serum and the radioactivity in the pellets was measured. The radioactivity obtained after adding 500-fold excess of unlabelled IgG2a (OKT3) was assumed to be due to nonspecific binding and was subtracted before calculating the percentage inhibitions shown. Radiolabelling was performed as described above.

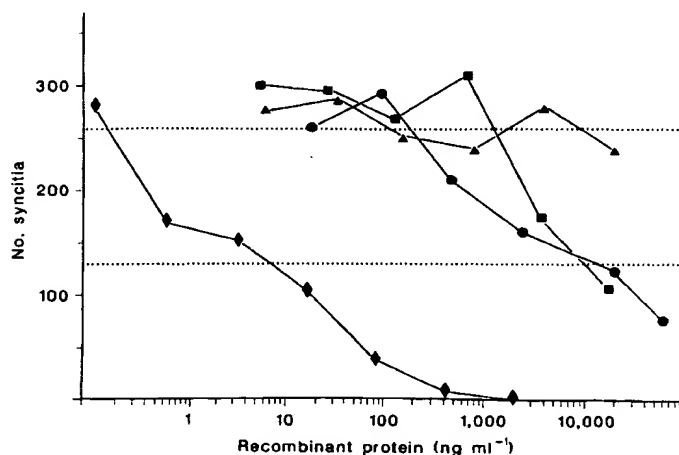


FIG. 4 Inhibition of syncytium formation by different recombinant proteins. The number of syncytia is plotted against the final concentrations of the recombinant proteins (●) CD4-M μ ; (■) CD4-M $\gamma 2a$; (●) CD4-M κ ; (▲) L3T4-M κ (ref. 9). The dotted lines indicate the number of syncytia in the absence of any inhibitory proteins and the number at 50% inhibition.

METHODS. A pretitrated amount of HIV-1-HAN was incubated at room temperature with serially diluted recombinant proteins for 30 min. Thereafter, $25 \mu\text{l}$ of each mixture was transferred in triplicate into the wells of a 96-well plate which contained 25,000 MT-2 cells³⁵ per well in $50 \mu\text{l}$ of medium. After 3 days culture, $100 \mu\text{l}$ of fresh medium was added per well and after 5 days the syncytia were counted. The sums of syncytia of the triplicates were used for inhibition curves. HIV-1-HAN has been isolated from the PBL of an AIDS patient. Partially determined nucleotide sequence of HIV-1-HAN shows about 90% sequence homology to HTLV-III_B (U. Sauer-mann and J. Mous, unpublished observation). Culture supernatants of MT-2 or Jurkat cells infected with HIV-1-HAN contained ten times more syncytium-forming capacity than similar culture supernatants of H9/HTLV-III_B cells.

in the regions of the CH2 domain responsible for C1q and Fc γ receptor binding.^{15,16}

To assay the biological activity of these molecules, we tested them in the syncytium inhibition assay^{17,18} (Fig. 4). CD4-M μ was by far the best inhibitor of syncytium formation: 50% inhibition was obtained at a concentration of 10 ng ml^{-1} which was about 1,000-fold less than the concentration ($\sim 10 \mu\text{g ml}^{-1}$) of CD4-M $\gamma 2a$ and CD4-M κ proteins needed for the same effect. Complete abolition of syncytia formation was possible with CD4-M μ at concentrations of about $1\text{--}2 \mu\text{g ml}^{-1}$. The CD4-M κ protein exists as a noncovalently-associated dimer (data not shown), possibly due to the $C\kappa$ portion of the molecules. Thus it seems that the effectiveness of these molecules increases as a function of their valence. Truly monovalent CD4, which we do not have, has not been compared directly with the dimeric forms of recombinant CD4 molecules (CD4-M $\gamma 2a$ and CD4-M κ) in this particular assay.

Soluble CD4 provides the optimal specificity for neutralization of the HIV-1 for many reasons. First, the strength of the interaction of the CD4 and gp120 is very high, of the order of 10^{-9} M (refs 5, 19). Second, the genetic variants of HIV-1 (refs 20–23) which emerge frequently during the infection must retain their CD4-binding properties to maintain their infectivity. Third, the immunity against gp120 acquired during the infection can have serious deleterious effects on the immune system: free gp120 which is shed from the virus^{24,25} can be trapped specifically on the CD4-positive cells and in this way the non-infected cells can become targets of various forms of anti-gp120 immune attacks^{26–29}. Passive immunity based on CD4 specificity would avoid this bystander destruction because it would discriminate between gp120 molecules which are already bound on cell-surface CD4 and those molecules which are produced by infected cells.

Although the human CD4-immunoglobulin chimera reported by Capon *et al.*¹⁰ was secreted, they found that a similar hybrid protein, based on the mouse γ_1 heavy chain, was retained intracellularly. We have also noticed that hybrid molecules containing the C_H1 domain, for example CD4-IgM chimaeras, are not secreted (unpublished observation) and we suspect that in the absence of immunoglobulin light chains, the hydrophobic face of the C_H1 domain interacts strongly with the heavy chain

binding protein, thus preventing secretion^{30,31}.

We believe that hybrid proteins which combine the specificity of CD4 with the multivalency and effector functions of different immunoglobulin subclasses could provide a realistic approach to AIDS therapy. We also think that our approach to designing hybrid immunoglobulin molecules could be applied more generally for building novel immunoglobulin molecules. □

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Activation of HIV gene expression during monocyte differentiation by induction of NF- κ B

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THE latent period of AIDS is influenced by factors which activate human immunodeficiency virus (HIV) replication in different cell types. Although monocytic cells may provide a reservoir for virus production *in vivo*¹⁻⁸, their regulation of HIV transcription has not been defined. We now report that HIV gene expression in the monocyte lineage is regulated by NF- κ B, the same transcription factor known to stimulate the HIV enhancer in activated T cells⁹; however, control of NF- κ B and HIV in monocytes differs from that observed in T cells. NF- κ B-binding activity appears during the transition from promonocyte to monocyte in U937 cells induced to differentiate *in vitro* and is present constitutively in mature monocytes and macrophages. In a chronically infected promonocytic cell, U1, differentiation is associated with HIV-1 replication as well as NF- κ B binding activity. These findings suggest that NF- κ B binding activity is developmentally regulated in the monocyte lineage, and that it provides one signal for HIV activation in these cells.

We transfected monocytic cell lines from progressive stages of differentiation with a plasmid containing the HIV enhancer linked to the chloroamphenicol acetyltransferase (CAT) gene. Twenty-four hours after transfection, cells were incubated in medium alone or in the presence of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Expression of the HIV enhancer was induced by TPA in two immature monocyte leukaemia lines, a human granulocyte-macrophage leukaemia, HL-60, and the human promonocytic line, U937 (Fig. 1a). TPA treatment did not augment CAT expression in the mature macrophage leukaemic cells, THP-1, P388D1, or PU5-1.8, which showed higher basal activity (Fig. 1b; note scale changes).

Using a mutant HIV-CAT plasmid containing alterations in both κ B sites⁹, we showed that induction of HIV-CAT expression in the immature lines, HL-60 and U937 (Fig. 1a), and constitutive expression in the mature lines was dependent on the κ B sites (Fig. 1b). This suggested that NF- κ B is present in the induced progenitors and in the mature cells, and we therefore looked for NF- κ B binding activity in nuclear extracts from these cell lines. NF- κ B binding activity in the immature lines, HL-60 and U937, was induced by TPA, whereas in the mature macrophage lines, THP-1, PU5-1.8, and P388D1, it was constitutively expressed (Fig. 2a). We then determined whether NF- κ B binding activity is present in normal human monocytes and/or macrophages. Nuclear extracts were prepared from human peripheral blood monocytes or adherent mononuclear cells, and NF- κ B binding activity was found in both cell types as well as in mouse peritoneal macrophages (Fig. 2b). NF- κ B binding is therefore constitutively active in normal and neoplastic mature mononuclear phagocytes, including blood monocytes and adherent macrophages.

Treatment of immature monocytes with TPA, or the water-soluble phorbol-12, 13-dibutyrate¹⁰ (PDB) (which is more easily removed from cells) causes differentiation into mature monocytes and macrophages, as judged by changes in cell growth, morphology, surface glycoproteins, and phagocytic function (Fig. 3, see also refs 11-15). HL-60 cells treated with PDB acquired characteristics of mature macrophages, displaying growth arrest, increased phagocytosis, adherence, FcR and Mo 1 expression. At the same time, these cells began to express NF- κ B binding activity which persisted even two days after

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United States Patent [19]

Smith et al.

[11] **Patent Number:** 5,395,760[45] **Date of Patent:** Mar. 7, 1995

- [54] **DNA ENCODING TUMOR NECROSIS FACTOR- α AND - β RECEPTORS**
- [75] **Inventors:** Craig A. Smith; Raymond G. Goodwin, both of Seattle; M. Patricia Beckmann, Poulsbo, all of Wash.
- [73] **Assignee:** Immunex Corporation, Seattle, Wash.
- [21] **Appl. No.:** 523,635
- [22] **Filed:** May 10, 1990

Related U.S. Application Data

- [63] Continuation-in-part of Ser. No. 421,417, Oct. 13, 1989, abandoned, which is a continuation-in-part of Ser. No. 405,370, Sep. 11, 1989, abandoned, which is a continuation-in-part of Ser. No. 403,241, Sep. 5, 1989, abandoned.
- [51] **Int. Cl.⁶** A61K 45/05; C12P 21/06; C12N 15/00; C07H 17/00
- [52] **U.S. Cl.** 435/240.1; 424/85.1; 435/69.4; 435/172.3; 530/351; 530/388.23; 536/23.51
- [58] **Field of Search** 536/23.51; 435/69.1, 435/69.4, 240.1; 424/85.1, 85.2, 85.8; 530/351, 388.23; 436/501

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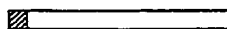
Primary Examiner—David L. Lacey
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Attorney, Agent, or Firm—Stephen L. Malaska;
 Christopher L. Wight

[57] **ABSTRACT**

Tumor necrosis factor receptor proteins, DNAs and expression vectors encoding TNF receptors, and processes for producing TNF receptors as products of recombinant cell culture, are disclosed.

20 Claims, 6 Drawing Sheets

HuTNF-R



HuTNF-RA235



HuTNF-RA185



HuTNF-RA163



HuTNF-RA142



MuTNF-R

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Figure 1

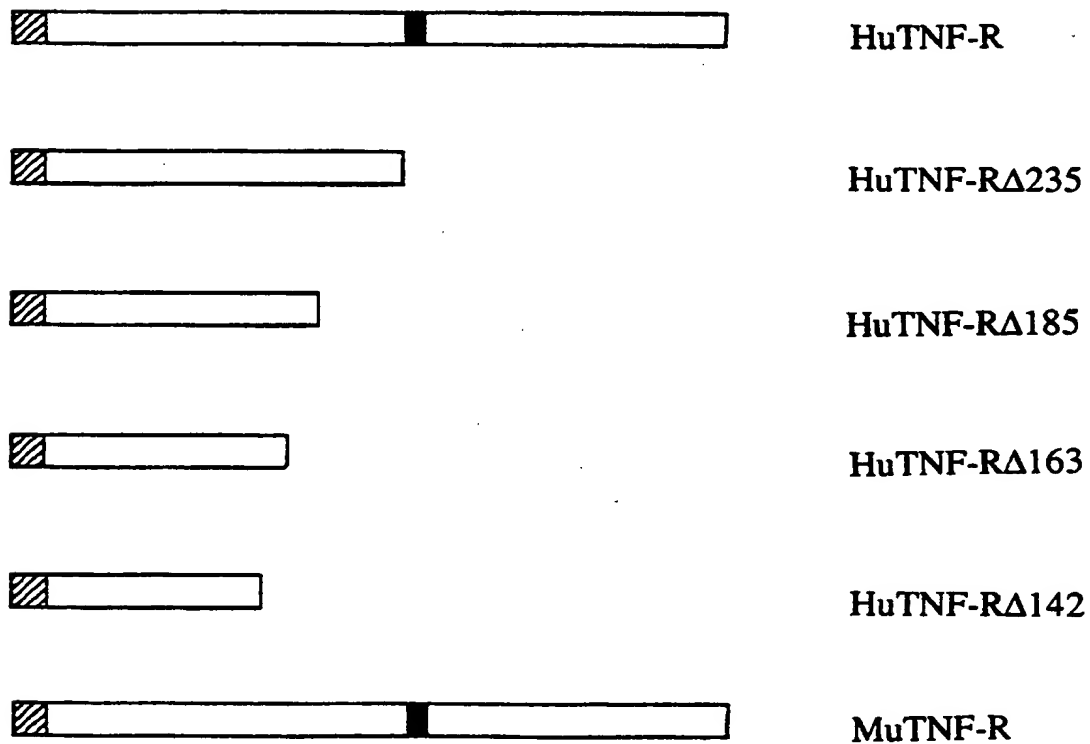


Figure 2A

GCGAGGCAGGCAGCCTGGAGAGAAGGCG	28
CTGGGCTGCGAGGGCGCGAGGGCGCGAGGGCAGGGGGCAACCGGACCCCGCCCGCATCC	87
ATG GCG CCC GTC GCC GTC TGG GCC GCG CTG GCC GTC GGA CTG GAG	132
Met Ala Pro Val Ala Val Trp Ala Ala Leu Ala Val Gly Leu Glu	-8
CTC TGG GCT GCG GCG CAC GCC TTG CCC GCC CAG GTG GCA TTT ACA	177
Leu Trp Ala Ala Ala His Ala Leu Pro Ala Gln Val Ala Phe Thr	8
CCC TAC GCC CCG GAG CCC GGG AGC ACA TGC CGG CTC AGA GAA TAC	222
Pro Tyr Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr	23
TAT GAC CAG ACA GCT CAG ATG TGC TGC AGC AAA TGC TCG CCG GGC	267
Tyr Asp Gln Thr Ala Gln Met Cys Cys Ser Lys Cys Ser Pro Gly	38
CAA CAT GCA AAA GTC TTC TGT ACC AAG ACC TCG GAC ACC GTG TGT	312
Gln His Ala Lys Val Phe Cys Thr Lys Thr Ser Asp Thr Val Cys	53
GAC TCC TGT GAG GAC AGC ACA TAC ACC CAG CTC TGG AAC TGG GTT	357
Asp Ser Cys Glu Asp Ser Thr Tyr Thr Gln Leu Trp Asn Trp Val	68
CCC GAG TGC TTG AGC TGT GGC TCC CGC TGT AGC TCT GAC CAG GTG	402
Pro Glu Cys Leu Ser Cys Gly Ser Arg Cys Ser Ser Asp Gln Val	83
GAA ACT CAA GCC TGC ACT CGG GAA CAG AAC CGC ATC TGC ACC TGC	447
Glu Thr Gln Ala Cys Thr Arg Glu Gln Asn Arg Ile Cys Thr Cys	98
AGG CCC GGC TGG TAC TGC GCG CTG AGC AAG CAG GAG GGG TGC CGG	492
Arg Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys Arg	113
CTG TGC GCG CCG CTG CGC AAG TGC CGC CCG GGC TTC GGC GTG GCC	537
Leu Cys Ala Pro Leu Arg Lys Cys Arg Pro Gly Phe Gly Val Ala	128
AGA CCA GGA ACT GAA ACA TCA GAC GTG GTG TGC AAG CCC TGT GCC	582
Arg Pro Gly Thr Glu Thr Ser Asp Val Val Cys Lys Pro Cys Ala	143
CCG GGG ACG TTC TCC AAC ACG ACT TCA TCC ACG GAT ATT TGC AGG	627
Pro Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr Asp Ile Cys Arg	158
CCC CAC CAG ATC TGT AAC GTG GTG GCC ATC CCT GGG AAT GCA AGC	672
Pro His Gln Ile Cys Asn Val Val Ala Ile Pro Gly Asn Ala Ser	173
ATG GAT GCA GTC TGC ACG TCC ACG TCC CCC ACC CGG AGT ATG GCC	717
Met Asp Ala Val Cys Thr Ser Thr Ser Pro Thr Arg Ser Met Ala	188
CCA GGG GCA GTA CAC TTA CCC CAG CCA GTG TCC ACA CGA TCC CAA	762
Pro Gly Ala Val His Leu Pro Gln Pro Val Ser Thr Arg Ser Gln	203
CAC ACG CAG CCA ACT CCA GAA CCC AGC ACT GCT CCA AGC ACC TCC	807
His Thr Gln Pro Thr Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser	218
TTC CTG CTC CCA ATG GGC CCC AGC CCC CCA GCT GAA GGG AGC ACT	852
Phe Leu Leu Pro Met Gly Pro Ser Pro Pro Ala Glu Gly Ser Thr	233
GGC GAC TTC GCT CTT CCA GTT GGA CTG ATT GTG GGT GTG ACA GCC	897
Gly Asp <u>Phe Ala Leu Pro Val Gly Leu Ile Val Gly Val Thr Ala</u>	248
TTG GGT CTA CTA ATA ATA GGA GTG GTG AAC TGT GTC ATC ATG ACC	942
<u>Leu Gly Leu Leu Ile Ile Gly Val Val Asn Cys Val Ile Met Thr</u>	263

Figure 2B

CAG GTG AAA AAG AAG CCC TTG TGC CTG CAG AGA GAA GCC AAG GTG	987
<u>Gln Val</u> Lys Lys Lys Pro Leu Cys Leu Gln Arg Glu Ala Lys Val	278
CCT CAC TTG CCT GCC GAT AAG GCC CGG GGT ACA CAG GGC CCC GAG	1032
Pro His Leu Pro Ala Asp Lys Ala Arg Gly Thr Gln Gly Pro Glu	293
CAG CAG CAC CTG CTG ATC ACA GCG CCG AGC TCC AGC AGC AGC TCC	1077
Gln Gln His Leu Leu Ile Thr Ala Pro Ser Ser Ser Ser Ser Ser	308
CTG GAG AGC TCG GCC AGT GCG TTG GAC AGA AGG GCG CCC ACT CGG	1122
Leu Glu Ser Ser Ala Ser Ala Leu Asp Arg Arg Ala Pro Thr Arg	323
AAC CAG CCA CAG GCA CCA GGC GTG GAG GCC AGT GGG GCC GGG GAG	1167
Asn Gln Pro Gln Ala Pro Gly Val Glu Ala Ser Gly Ala Gly Glu	338
GCC CGG GCC AGC ACC GGG AGC TCA GAT TCT TCC CCT GGT GGC CAT	1212
Ala Arg Ala Ser Thr Gly Ser Ser Asp Ser Ser Pro Gly Gly His	353
GGG ACC CAG GTC AAT GTC ACC TGC ATC GTG AAC GTC TGT AGC AGC	1257
Gly Thr Gln Val Asn Val Thr Cys Ile Val Asn Val Cys Ser Ser	368
TCT GAC CAC AGC TCA CAG TGC TCC TCC CAA GCC AGC TCC ACA ATG	1302
Ser Asp His Ser Ser Gln Cys Ser Ser Gln Ala Ser Ser Thr Met	383
GGA GAC ACA GAT TCC AGC CCC TCG GAG TCC CCG AAG GAC GAG CAG	1347
Gly Asp Thr Asp Ser Ser Pro Ser Glu Ser Pro Lys Asp Glu Gln	398
GTC CCC TTC TCC AAG GAG GAA TGT GCC TTT CGG TCA CAG CTG GAG	1392
Val Pro Phe Ser Lys Glu Glu Cys Ala Phe Arg Ser Gln Leu Glu	413
ACG CCA GAG ACC CTG CTG GGG AGC ACC GAA GAG AAG CCC CTG CCC	1437
Thr Pro Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys Pro Leu Pro	428
CTT GGA GTG CCT GAT GCT GGG ATG AAG CCC AGT	1470
Leu Gly Val Pro Asp Ala Gly Met Lys Pro Ser	439
TAACCAGGCCGGTGTGGGCTGTGTCGTAGCCAAGGTGGGCTGAGCCCTGGCAGGATGAC	
CCTGCGAAGGGGCCCTGGTCCTTCCAGGCCCCCACCCTAGGACTCTGAGGCTCTTTCT	
GGGCCAAGTTCTCTAGTGCCCTCCACAGCCGCAGCCTCCCTCTGACCTGCAG...	

Figure 3A

CGCAGCTGAGGCACTAGAGCTCC																23
AGGCACAAGGGCGGGAGCCACCGCTGCCCT																75
Met Ala Pro Ala Ala Leu Trp																-16
GTC GCG CTG GTC TTC GAA CTG CAG CTG TGG GCC ACC GGG CAC ACA																120
Val Ala Leu Val Phe Glu Leu Gln Leu Trp Ala Thr Gly His Thr																-1
GTG CCC GCC CAG GTT GTC TTG ACA CCC TAC AAA CCG GAA CCT GGG																165
Val Pro Ala Gln Val Val Leu Thr Pro Tyr Lys Pro Glu Pro Gly																15
TAC GAG TGC CAG ATC TCA CAG GAA TAC TAT GAC AGG AAG GCT CAG																210
Tyr Glu Cys Gln Ile Ser Gln Glu Tyr Tyr Asp Arg Lys Ala Gln																30
ATG TGC TGT GCT AAG TGT CCT CCT GGC CAA TAT GTG AAA CAT TTC																255
Met Cys Cys Ala Lys Cys Pro Pro Gly Gln Tyr Val Lys His Phe																45
TGC AAC AAG ACC TCG GAC ACC GTG TGT GCG GAC TGT GAG GCA AGC																300
Cys Asn Lys Thr Ser Asp Thr Val Cys Ala Asp Cys Glu Ala Ser																60
ATG TAT ACC CAG GTC TGG AAC CAG TTT CGT ACA TGT TTG AGC TGC																345
Met Tyr Thr Gln Val Trp Asn Gln Phe Arg Thr Cys Leu Ser Cys																75
AGT TCT TCC TGT ACC ACT GAC CAG GTG GAG ATC CGC GCC TGC ACT																390
Ser Ser Ser Cys Thr Thr Asp Gln Val Glu Ile Arg Ala Cys Thr																90
AAA CAG CAG AAC CGA GTG TGT GCT TGC GAA GCT GGC AGG TAC TgC																435
Lys Gln Gln Asn Arg Val Cys Ala Cys Glu Ala Gly Arg Tyr Cys																105
GCC TTG AAA ACC CAT TCT GGC AGC TGT CGA CAG TGC ATG AGG CTG																480
Ala Leu Lys Thr His Ser Gly Ser Cys Arg Gln Cys Met Arg Leu																120
AGC AAG TGC GGC CCT GGC TTC GGA GTG GCC AGT TCA AGA GCC CCA																525
Ser Lys Cys Gly Pro Gly Phe Gly Val Ala Ser Ser Arg Ala Pro																135
AAT GGA AAT GTG CTA TGC AAG GCC TGT GCC CCA GGG ACG TTC TCT																570
Asn Gly Asn Val Leu Cys Lys Ala Cys Ala Pro Gly Thr Phe Ser																150
GAC ACC ACA TCA TCC ACT GAT GTG TGC AGG CCC CAC CGC ATC TGT																615
Asp Thr Thr Ser Ser Thr Asp Val Cys Arg Pro His Arg Ile Cys																165
AGC ATC CTG GCT ATT CCC GGA AAT GCA AGC ACA GAT GCA GTC TGT																660
Ser Ile Leu Ala Ile Pro Gly Asn Ala Ser Thr Asp Ala Val Cys																180
GCG CCC GAG TCC CCA ACT CTA AGT GCC ATC CCA AGG ACA CTC TAC																705
Ala Pro Glu Ser Pro Thr Leu Ser Ala Ile Pro Arg Thr Leu Tyr																195
GTA TCT CAG CCA GAG CCC ACA AGA TCC CAA CCC CTG GAT CAA GAG																750
Val Ser Gln Pro Glu Pro Thr Arg Ser Gln Pro Leu Asp Gln Glu																210
CCA GGG CCC AGC CAA ACT CCA AGC ATC CTT ACA TCG TTG GGT TCA																795
Pro Gly Pro Ser Gln Thr Pro Ser Ile Leu Thr Ser Leu Gly Ser																225
ACC CCC ATT ATT GAA CAA AGT ACC AAG GGT GGC ATC TCT CTT CCA																840
Thr Pro Ile Ile Glu Gln Ser Thr Lys Gly Gly Ile Ser Leu Pro																240
ATT GGT CTG ATT GTT GGA GTG ACA TCA CTG GGT CTG CTG ATG TTA																885
Ile Gly Leu Ile Val Gly Val Thr Ser Leu Gly Leu Leu Met Leu																255

Figure 3B

GGA CTG GTG AAC TGC ATC ATC CTG GTG CAG AGG AAA AAG AAG CCC	930
Gly Leu Val Asn Cys Ile Ile Leu Val Gln Arg Lys Lys Lys Pro	270
TCC TGC CTA CAA AGA GAT GCC AAG GTG CCT CAT GTG CCT GAT GAG	975
Ser Cys Leu Gln Arg Asp Ala Lys Val Pro His Val Pro Asp Glu	285
AAA TCC CAG GAT GCA GTA GGC CTT GAG CAG CAG CAC CTG TTG ACC	1020
Lys Ser Gln Asp Ala Val Gly Leu Glu Gln Gln His Leu Leu Thr	300
ACA GCA CCC AGT TCC AGC AGC AGC TCC CTA GAG AGC TCA GCC AGC	1065
Thr Ala Pro Ser Ser Ser Ser Ser Ser Leu Glu Ser Ser Ala Ser	315
GCT GGG GAC CGA AGG GCG CCC CCT GGG GGC CAT CCC CAA GCA AGA	1110
Ala Gly Asp Arg Arg Ala Pro Pro Gly Gly His Pro Gln Ala Arg	330
GTC ATG GCG GAG GCC CAA GGG TTT CAG GAG GCC CGT GCC AGC TCC	1155
Val Met Ala Glu Ala Gln Gly Phe Gln Glu Ala Arg Ala Ser Ser	345
AGG ATT TCA GAT TCT TCC CAC GGA AGC CAC GGG ACC CAC GTC AAC	1200
Arg Ile Ser Asp Ser Ser His Gly Ser His Gly Thr His Val Asn	360
GTC ACC TGC ATC GTG AAC GTC TGT AGC AGC TCT GAC CAC AGT TCT	1245
Val Thr Cys Ile Val Asn Val Cys Ser Ser Ser Asp His Ser Ser	375
CAG TGC TCT TCC CAA GCC AGC GCC ACA GTG GGA GAC CCA GAT GCC	1290
Gln Cys Ser Ser Gln Ala Ser Ala Thr Val Gly Asp Pro Asp Ala	390
AAG CCC TCA GCG TCC CCA AAG GAT GAG CAG GTC CCC TTC TCT CAG	1335
Lys Pro Ser Ala Ser Pro Lys Asp Glu Gln Val Pro Phe Ser Gln	405
GAG GAG TGT CCG TCT CAG TCC CCG TGT GAG ACT ACA GAG ACA CTG	1380
Glu Glu Cys Pro Ser Gln Ser Pro Cys Glu Thr Thr Glu Thr Leu	420
CAG AGC CAT GAG AAG CCC TTG CCC CTT GGT GTG CCG GAT ATG GGC	1425
Gln Ser His Glu Lys Pro Leu Pro Leu Gly Val Pro Asp Met Gly	435
ATG AAG CCC AGC CAA GCT GGC TGG TTT GAT CAG ATT GCA GTC AAA	1470
Met Lys Pro Ser Gln Ala Gly Trp Phe Asp Gln Ile Ala Val Lys	450
GTG GCC	1476
Val Ala	452
TGACCCCTGACAGGGGTAACACCCTGCAAAGGGACCCCGAGACCCTGAACCCATGGAAC	1536
TTCATGACTTTTGCTGGATCCATTTCCCTTAGTGGCTTCCAGAGCCCCAGTTGAGGTCA	1596
AGTGAGGGGCTGAGACAGCTAGAGTGGTCAAAAAGTCCATGGTGTTTTATGGGGCAGTC	1656
CCAGGAAGTTGTTGCTCTTCCATGACCCCTCTGGATCTCCTGGGCTCTTGCCTGATTCTT	1716
GCTTCTGAGAGGCCCCAGTATTTTTCTTCTAAGGAGCTAACATCCTCTTCCATGAATA	1776
GCACAGCTCTTCAGCCTGAATGCTGACACTGCAGGGCGGTTCCAGCAAGTAGGAGCAAGT	1836
GGTGGCCTGGTAGGGCACAGAGGCCCTTCAGGTTAGTGCTAAACTCTTAGGAAGTACCCT	1896
CTCCAAGCCACCGAAATTCTTTTGATGCAAGAATCAGAGGCCCATCAGGCAGAGTTGC	1956
TCTGTTATAGGATGGTAGGGCTGTAACTCAGTGGTCCAGTGTGCTTTTAGCATGCCCTGG	2016
GTTTGATCCTCAGCAACACATGCAAAACGTAAGTAGACAGCAGACAGCAGACAGCAGC	2076
CAGCCCCCTGTGTGGTTGCAGCCTCTGCCTTTTACTCTGGTGGGCACACAGAG	2136
GGCTGGAGCTCCTCCTGACCTTCTAATGAGCCCTTCCAAGGCCACGCCTTCCCTCAG	2196
GGAATCTCAGGGACTGTAGAGTTCCCAGGCCCTGCAGCCACCTGTCTCTTCCCTACCTCA	2256
GCCTGGAGCACTCCCTCTAACTCCCCAACGGCTTGGTACTGTACTTGTGTGACCCCAAC	2316
GTGCATTGTCCGGGTTAGGCACTGTGAGTTGGAACAGCTCATGACATCGGTTGAAAGGCC	2376
CACCCGGAAACAGCTAAGCCAGCTCTTTTGCCAAAGGATTGATGCCGGTTTTCTAATCA	2436
CCTGCTCCCTAGCATTTGCCTGGAAGGAAAGGTTTCAGGAGACTCCTCAAGAAGCAAGTTC	2496
AGTCTCAGGTGCTTGATGCCATGCTCACCGATTCCACTGGATATGAACTTGGCAGAGGA	2556

Figure 3C

[illegible]

DNA ENCODING TUMOR NECROSIS FACTOR- α AND - β RECEPTORS

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of U.S. application Ser. No. 421,417, filed Oct. 13, 1989, now abandoned, which is a continuation-in-part of U.S. application Ser. No. 405,370, filed Sep. 11, 1989, now abandoned, which is a continuation-in-part of U.S. application Ser. No. 403,241, filed Sep. 5, 1989, now abandoned.

BACKGROUND OF THE INVENTION

The present invention relates generally to cytokine receptors and more specifically to tumor necrosis factor receptors.

Tumor necrosis factor- α (TNF α , also known as cachectin) and tumor necrosis factor- β (TNF β , also known as lymphotoxin) are homologous mammalian endogenous secretory proteins capable of inducing a wide variety of effects on a large number of cell types. The great similarities in the structural and functional characteristics of these two cytokines have resulted in their collective description as "TNF." Complementary cDNA clones encoding TNF α (Pennica et al., *Nature* 312:724, 1984) and TNF β (Gray et al., *Nature* 312:721, 1984) have been isolated, permitting further structural and biological characterization of TNF.

TNF proteins initiate their biological effect on cells by binding to specific TNF receptor (TNF-R) proteins expressed on the plasma membrane of a TNF-responsive cell. TNF α and TNF β were first shown to bind to a common receptor on the human cervical carcinoma cell line ME-180 (Aggarwal et al., *Nature* 318:665, 1985). Estimates of the size of the TNF-R determined by affinity labeling studies ranged from 54 to 175 kDa (Creasey et al., *Proc. Natl. Acad. Sci. USA* 84:3293, 1987; Stauber et al., *J. Biol. Chem.* 263:19098, 1988; Hohmann et al., *J. Biol. Chem.* 264:14927, 1989). Although the relationship between these TNF-Rs of different molecular mass is unclear, Hohmann et al. (*J. Biol. Chem.* 264:14927, 1989) reported that at least two different cell surface receptors for TNF exist on different cell types. These receptors have an apparent molecular mass of about 80 kDa and about 55–60 kDa, respectively. None of the above publications, however, reported the purification to homogeneity of cell surface TNF receptors.

In addition to cell surface receptors for TNF, soluble proteins from human urine capable of binding TNF have also been identified (Peetre et al., *Eur. J. Haematol.* 41:414, 1988; Seckinger et al., *J. Exp. Med.* 167:1511, 1988; Seckinger et al., *J. Biol. Chem.* 264:11966, 1989; UK Patent Application, Publ. No. 2 218 101 A to Seckinger et al.; Engelmann et al., *J. Biol. Chem.* 264:11974, 1989). The soluble urinary TNF binding protein disclosed by UK 2 218 101 A has a partial N-terminal amino acid sequence of Asp—Ser—Val—Cys—Pro—, which corresponds to the partial sequence disclosed later by Engelmann et al. (1989). The relationship of the above soluble urinary binding proteins was further elucidated after original parent application (U.S. Ser. No. 403,241) of the present application was filed, when Engelmann et al. reported the identification and purification of a second distinct soluble urinary TNF binding protein having an N-terminal amino acid sequence of Val—Ala—Phe—Thr—

Pro— (*J. Biol. Chem.* 265:1531, 1990). The two urinary proteins disclosed by the UK 2 218 101 A and the Engelmann et al. publications were shown to be immunologically related to two apparently distinct cell surface proteins by the ability of antiserum against the binding proteins to inhibit TNF binding to certain cells.

More recently, two separate groups reported the molecular cloning and expression of a human 55 kDa TNF-R (Loetscher et al., *Cell* 61:351, 1990; Schall et al., *Cell* 61:361, 1990). The TNF-R of both groups has an N-terminal amino acid sequence which corresponds to the partial amino acid sequence of the urinary binding protein disclosed by UK 2 218 101 A, Engelmann et al. (1989) and Engelmann et al. (1990).

In order to elucidate the relationship of the multiple forms of TNF-R and soluble urinary TNF binding proteins, or to study the structural and biological characteristics of TNF-Rs and the role played by TNF-Rs in the responses of various cell populations to TNF or other cytokine stimulation, or to use TNF-Rs effectively in therapy, diagnosis, or assay, purified compositions of TNF-R are needed. Such compositions, however, are obtainable in practical yields only by cloning and expressing genes encoding the receptors using recombinant DNA technology. Effort to purify the TNF-R molecule for use in biochemical analysis or to clone and express mammalian genes encoding TNF-R, however, have been impeded by lack of a suitable source of receptor protein or mRNA. Prior to the present invention, no cell lines were known to express high levels of TNF-R constitutively and continuously, which precluded purification of receptor for sequencing or construction of genetic libraries for cDNA cloning.

SUMMARY OF THE INVENTION

The present invention provides isolated TNF receptors and DNA sequences encoding mammalian tumor necrosis factor receptors (TNF-R), in particular, human TNF-Rs. Such DNA sequences include (a) cDNA clones having a nucleotide sequence derived from the coding region of a native TNF-R gene; (b) DNA sequences which are capable of hybridization to the cDNA clones of (a) under moderately stringent conditions and which encode biologically active TNF-R molecules; or (c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode biologically active TNF-R molecules. In particular, the present invention provides DNA sequences which encode soluble TNF receptors.

The present invention also provides recombinant expression vectors comprising the DNA sequences defined above, recombinant TNF-R molecules produced using the recombinant expression vectors, and processes for producing the recombinant TNF-R molecules using the expression vectors.

The present invention also provides isolated or purified protein compositions comprising TNF-R, and, in particular, soluble forms of TNF-R.

The present invention also provides compositions for use in therapy, diagnosis, assay of TNF-R, or in raising antibodies to TNF-R, comprising effective quantities of soluble native or recombinant receptor proteins prepared according to the foregoing processes.

Because of the ability of TNF to specifically bind TNF receptors (TNF-Rs), purified TNF-R composi-

tions will be useful in diagnostic assays for TNF, as well as in raising antibodies to TNF receptor for use in diagnosis and therapy. In addition, purified TNF receptor compositions may be used directly in therapy to bind or scavenge TNF, thereby providing a means for regulating the immune activities of this cytokine.

These and other aspects of the present invention will become evident upon reference to the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of the coding region of various cDNAs encoding human and murine TNF-Rs. The leader sequence is hatched and the transmembrane region is solid.

FIGS. 2A-2B depict the partial cDNA sequence and derived amino acid sequence of the human TNF-R clone 1. Nucleotides are numbered from the beginning of the 5' untranslated region. Amino acids are numbered from the beginning of the signal peptide sequence. The putative signal peptide sequence is represented by the amino acids -22 to -1. The N-terminal leucine of the mature TNF-R protein is underlined at position 1. The predicted transmembrane region from amino acids 236 to 265 is also underlined. The C-termini of various soluble TNF-Rs are marked with an arrow (\uparrow).

FIGS. 3A-3C depict the cDNA sequence and derived amino acid sequence of murine TNF-R clone 11. The putative signal peptide sequence is represented by amino acids -22 to -1. The N-terminal valine of the mature TNF-R protein is underlined at position 1. The predicted transmembrane region from amino acids 234 to 265 is also underlined.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, the terms "TNF receptor" and "TNF-R" refer to proteins having amino acid sequences which are substantially similar to the native mammalian TNF receptor amino acid sequences, and which are biologically active, as defined below, in that they are capable of binding TNF molecules or transducing a biological signal initiated by a TNF molecule binding to a cell, or cross-reacting with anti-TNF-R antibodies raised against TNF-R from natural (i.e., nonrecombinant) sources. The mature full-length human TNF-R is a glycoprotein having a molecular weight of about 80 kilodaltons (kDa). As used throughout the specification, the term "mature" means a protein expressed in a form lacking a leader sequence as may be present in full-length transcripts of a native gene. Experiments using COS cells transfected with a cDNA encoding full-length human TNF-R showed that TNF-R bound ^{125}I -TNF α with an apparent K_d of about $5 \times 10^9 \text{M}^{-1}$, and that TNF-R bound ^{125}I -TNF β with an apparent K_d of about $2 \times 10^9 \text{M}^{-1}$. The terms "TNF receptor" or "TNF-R" include, but are not limited to, analogs or subunits of native proteins having at least 20 amino acids and which exhibit at least some biological activity in common with TNF-R, for example, soluble TNF-R constructs which are devoid of a transmembrane region (and are secreted from the cell) but retain the ability to bind TNF. Various bioequivalent protein and amino acid analogs are described in detail below.

The nomenclature for TNF-R analogs as used herein follows the convention of naming the protein (e.g., TNF-R) preceded by either hu (for human) or mu (for

murine) and followed by a Δ (to designate a deletion) and the number of the C-terminal amino acid. For example, huTNF-RA235 refers to human TNF-R having Asp²³⁵ as the C-terminal amino acid (i.e., a polypeptide having the sequence of amino acids 1-235 of FIG. 2A). In the absence of any human or murine species designation, TNF-R refers generically to mammalian TNF-R. Similarly, in the absence of any specific designation for deletion mutants, the term TNF-R means all forms of TNF-R, including mutants and analogs which possess TNF-R biological activity.

"Soluble TNF-R" or "sTNF-R" as used in the context of the present invention refer to proteins, or substantially equivalent analogs, having an amino acid sequence corresponding to all or part of the extracellular region of a native TNF-R, for example, huTNF-RA235, huTNF-RA185 and huTNF-RA163, or amino acid sequences substantially similar to the sequences of amino acids 1-163, amino acids 1-185, or amino acids 1-235 of FIG. 2A, and which are biologically active in that they bind to TNF ligand. Equivalent soluble TNF-Rs include polypeptides which vary from these sequences by one or more substitutions, deletions, or additions, and which retain the ability to bind TNF or inhibit TNF signal transduction activity via cell surface bound TNF receptor proteins, for example huTNF-RA x , wherein x is selected from the group consisting of any one of amino acids 163-235 of FIG. 2A. Analogous deletions may be made to muTNF-R. Inhibition of TNF signal transduction activity can be determined by transfecting cells with recombinant TNF-R DNAs to obtain recombinant receptor expression. The cells are then contacted with TNF and the resulting metabolic effects examined. If an effect results which is attributable to the action of the ligand, then the recombinant receptor has signal transduction activity. Exemplary procedures for determining whether a polypeptide has signal transduction activity are disclosed by Idzerda et al., *J. Exp. Med.* 171:861 (1990); Curtis et al., *Proc. Natl. Acad. Sci. USA* 86:3045 (1989); Prywes et al., *EMBO J.* 5:2179 (1986) and Chou et al., *J. Biol. Chem.* 262:1842 (1987). Alternatively, primary cells or cell lines which express an endogenous TNF receptor and have a detectable biological response to TNF could also be utilized.

The term "isolated" or "purified", as used in the context of this specification to define the purity of TNF-R protein or protein compositions, means that the protein or protein composition is substantially free of other proteins of natural or endogenous origin and contains less than about 1% by mass of protein contaminants residual of production processes. Such compositions, however, can contain other proteins added as stabilizers, carriers, excipients or co-therapeutics. TNF-R is isolated if it is detectable as a single protein band in a polyacrylamide gel by silver staining.

The term "substantially similar," when used to define either amino acid or nucleic acid sequences, means that a particular subject sequence, for example, a mutant sequence, varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which is to retain biological activity of the TNF-R protein as may be determined, for example, in one of the TNF-R binding assays set forth in Example 1 below. Alternatively, nucleic acid subunits and analogs are "substantially similar" to the specific DNA sequences disclosed herein if: (a) the DNA sequence is derived from the coding region of a native mammalian TNF-R

gene; (b) the DNA sequence is capable of hybridization to DNA sequences of (a) under moderately stringent conditions (50° C., 2× SSC) and which encode biologically active TNF-R molecules; or DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) or (b) and which encode biologically active TNF-R molecules.

"Recombinant," as used herein, means that a protein is derived from recombinant (e.g., microbial or mammalian) expression systems. "Microbial" refers to recombinant proteins made in bacterial or fungal (e.g., yeast) expression systems. As a product, "recombinant microbial" defines a protein produced in a microbial expression system which is essentially free of native endogenous substances. Protein expressed in most bacterial cultures, e.g., *E. coli*, will be free of glycan. Protein expressed in yeast may have a glycosylation pattern different from that expressed in mammalian cells.

"Biologically active," as used throughout the specification as a characteristic of TNF receptors, means that a particular molecule shares sufficient amino acid sequence similarity with the embodiments of the present invention disclosed herein to be capable of binding detectable quantities of TNF, transmitting a TNF stimulus to a cell, for example, as a component of a hybrid receptor construct, or cross-reacting with anti-TNF-R antibodies raised against TNF-R from natural (i.e., non-recombinant) sources. Preferably, biologically active TNF receptors within the scope of the present invention are capable of binding greater than 0.1 nmoles TNF per nmole receptor, and most preferably, greater than 0.5 nmole TNF per nmole receptor in standard binding assays (see below).

"Isolated DNA sequence" refers to a DNA polymer, in the form of a separate fragment or as a component of a larger DNA construct, which has been derived from DNA isolated at least once in substantially pure form, i.e., free of contaminating endogenous materials and in a quantity or concentration enabling identification, manipulation, and recovery of the sequence and its component nucleotide sequences by standard biochemical methods, for example, using a cloning vector. Such sequences are preferably provided in the form of an open reading frame uninterrupted by internal nontranslated sequences, or introns, which are typically present in eukaryotic genes. Genomic DNA containing the relevant sequences could also be used as a source of coding sequences. Sequences of non-translated DNA may be present 5' or 3' from the open reading frame, where the same do not interfere with manipulation or expression of the coding regions.

"Nucleotide sequence" refers to a heteropolymer of deoxyribonucleotides. DNA sequences encoding the proteins provided by this invention can be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene which is capable of being expressed in a recombinant transcriptional unit.

Isolation of cDNAs Encoding TNF-R

The coding sequence of TNF-R is obtained by isolating a complementary DNA (cDNA) sequence encoding TNF-R from a recombinant cDNA or genomic DNA library. A cDNA library is preferably constructed by obtaining polyadenylated mRNA from a particular cell line which expresses a mammalian TNF-R, for example, the human fibroblast cell line WI-26 VA4 (ATCC CCL 95.1) and using the mRNA as a template for synthesiz-

ing double stranded cDNA. The double stranded cDNA is then packaged into a recombinant vector, which is introduced into an appropriate *E. coli* strain and propagated. Murine or other mammalian cell lines which express TNF-R may also be used. TNF-R sequences contained in the cDNA library can be readily identified by screening the library with an appropriate nucleic acid probe which is capable of hybridizing with TNF-R cDNA. Alternatively, DNAs encoding TNF-R proteins can be assembled by ligation of synthetic oligonucleotide subunits corresponding to all or part of the sequence of FIGS. 2A-2B or 3A-3C to provide a complete coding sequence.

The human TNF receptor cDNAs of the present invention were isolated by the method of direct expression cloning. A cDNA library was constructed by first isolating cytoplasmic mRNA from the human fibroblast cell line WI-26 VA4. Polyadenylated RNA was isolated and used to prepare double-stranded cDNA. Purified cDNA fragments were then ligated into pCAV/NOT vector DNA which uses regulatory sequences derived from pDC201 (a derivative of pMLSV, previously described by Cosman et al., *Nature* 312:768, 1984), SV40 and cytomegalovirus DNA, described in detail below in Example 2. pCAV/NOT has been deposited with the American Type Culture Collection under accession No. ATCC 68014. The pCAV/NOT vectors containing the WI26-VA4 cDNA fragments were transformed into *E. coli* strain DH5 α . Transformants were plated to provide approximately 800 colonies per plate. The resulting colonies were harvested and each pool used to prepare plasmid DNA for transfection into COS-7 cells essentially as described by Cosman et al. (*Nature* 312:768, 1984) and Luthman et al. (*Nucl. Acid Res.* 11:1295, 1983). Transformants expressing biologically active cell surface TNF receptors were identified by screening for their ability to bind ¹²⁵I-TNF. In this screening approach, transfected COS-7 cells were incubated with medium containing ¹²⁵I-TNF, the cells washed to remove unbound labeled TNF, and the cell monolayers contacted with X-ray film to detect concentrations of TNF binding, as disclosed by Sims et al., *Science* 241:585 (1988). Transfectants detected in this manner appear as dark foci against a relatively light background.

Using this approach, approximately 240,000 cDNAs were screened in pools of approximately 800 cDNAs until assay of one transfectant pool indicated positive foci for TNF binding. A frozen stock of bacteria from this positive pool was grown in culture and plated to provide individual colonies, which were screened until a single clone (clone 1) was identified which was capable of directing synthesis of a surface protein with detectable TNF binding activity. The sequence of cDNA clone 1 isolated by the above method is depicted in FIGS. 2A-2C.

Additional cDNA clones can be isolated from cDNA libraries of other mammalian species by cross-species hybridization. For use in hybridization, DNA encoding TNF-R may be covalently labeled with a detectable substance such as a fluorescent group, a radioactive atom or a chemiluminescent group by methods well known to those skilled in the art. Such probes could also be used for in vitro diagnosis of particular conditions.

Like most mammalian genes, mammalian TNF receptors are presumably encoded by multi-exon genes. Alternative mRNA constructs which can be attributed to different mRNA splicing events following transcription, and which share large regions of identity or simi-

larity with the cDNAs claimed herein, are considered to be within the scope of the present invention.

Other mammalian TNF-R cDNAs are isolated by using an appropriate human TNF-R DNA sequence as a probe for screening a particular mammalian cDNA library by cross-species hybridization.

Proteins and Analogs

The present invention provides isolated recombinant mammalian TNF-R polypeptides. Isolated TNF-R polypeptides of this invention are substantially free of other contaminating materials of natural or endogenous origin and contain less than about 1% by mass of protein contaminants residual of production processes. The native human TNF-R molecules are recovered from cell lysates as glycoproteins having an apparent molecular weight by SDS-PAGE of about 80 kilodaltons (kDa). The TNF-R polypeptides of this invention are optionally without associated native-pattern glycosylation.

Mammalian TNF-R of the present invention includes, by way of example, primate, human, murine, canine, feline, bovine, ovine, equine and porcine TNF-R. Mammalian TNF-Rs can be obtained by cross species hybridization, using a single stranded cDNA derived from the human TNF-R DNA sequence as a hybridization probe to isolate TNF-R cDNAs from mammalian cDNA libraries.

Derivatives of TNF-R within the scope of the invention also include various structural forms of the primary protein which retain biological activity. Due to the presence of ionizable amino and carboxyl groups, for example, a TNF-R protein may be in the form of acidic or basic salts, or may be in neutral form. Individual amino acid residues may also be modified by oxidation or reduction.

The primary amino acid structure may be modified by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like, or by creating amino acid sequence routants. Covalent derivatives are prepared by linking particular functional groups to TNF-R amino acid side chains or at the N- or C-termini. Other derivatives of TNF-R within the scope of this invention include covalent or aggregative conjugates of TNF-R or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugated peptide may be a signal (or leader) polypeptide sequence at the N-terminal region of the protein which co-translationally or post-translationally directs transfer of the protein from its site of synthesis to its site of function inside or outside of the cell membrane or wall (e.g., the yeast α -factor leader). TNF-R protein fusions can comprise peptides added to facilitate purification or identification of TNF-R (e.g., poly-His). The amino acid sequence of TNF receptor can also be linked to the peptide Asp—Tyr—Lys—Asp—Asp—Asp—Lys (DYKDDDDK) (Hopp et al., *Bio/Technology* 6:1204, 1988.) The latter sequence is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein. This sequence is also specifically cleaved by bovine mucosal enterokinase at the residue immediately following the Asp—Lys pairing. Fusion proteins capped with this peptide may also be resistant to intracellular degradation in *E. coli*.

TNF-R derivatives may also be used as immunogens, reagents in receptor-based immunoassays, or as binding agents for affinity purification procedures of TNF or other binding ligands. TNF-R derivatives may also be obtained by cross-linking agents, such as M-maleimido-benzoyl succinimide ester and N-hydroxysuccinimide, at cysteine and lysine residues. TNF-R proteins may also be covalently bound through reactive side groups to various insoluble substrates, such as cyanogen bromide-activated, bisoxirane-activated, carbonyl-diimidazole-activated or tosyl-activated agarose structures, or by adsorbing to polyolefin surfaces (with or without glutaraldehyde cross-linking). Once bound to a substrate, TNF-R may be used to selectively bind (for purposes of assay or purification) anti-TNF-R antibodies or TNF.

The present invention also includes TNF-R with or without associated native-pattern glycosylation. TNF-R expressed in yeast or mammalian expression systems, e.g., COS-7 cells, may be similar or slightly different in molecular weight and glycosylation pattern than the native molecules, depending upon the expression system. Expression of TNF-R DNAs in bacteria such as *E. coli* provides non-glycosylated molecules. Functional mutant analogs of mammalian TNF-R having inactivated N-glycosylation sites can be produced by oligonucleotide synthesis and ligation or by site-specific mutagenesis techniques. These analog proteins can be produced in a homogeneous, reduced-carbohydrate form in good yield using yeast expression systems. N-glycosylation sites in eukaryotic proteins are characterized by the amino acid triplet Asn—A₁—Z, where A₁ is any amino acid except Pro, and Z is Ser or Thr. In this sequence, asparagine provides a side chain amino group for covalent attachment of carbohydrate. Such a site can be eliminated by substituting another amino acid for Asn or for residue Z, deleting Asn or Z, or inserting a non-Z amino acid between A₁ and Z, or an amino acid other than Asn between Asn and A₁.

TNF-R derivatives may also be obtained by mutations of TNF-R or its subunits. A TNF-R mutant, as referred to herein, is a polypeptide homologous to TNF-R but which has an amino acid sequence different from native TNF-R because of a deletion, insertion or substitution.

Bioequivalent analogs of TNF-R proteins may be constructed by, for example, making various substitutions of residues or sequences or deleting terminal or internal residues or sequences not needed for biological activity. For example, cysteine residues can be deleted (e.g., Cys¹⁷⁸) or replaced with other amino acids to prevent formation of unnecessary or incorrect intramolecular disulfide bridges upon renaturation. Other approaches to mutagenesis involve modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. Generally, substitutions should be made conservatively; i.e., the most preferred substitute amino acids are those having physiochemical characteristics resembling those of the residue to be replaced. Similarly, when a deletion or insertion strategy is adopted, the potential effect of the deletion or insertion on biological activity should be considered. Substantially similar polypeptide sequences, as defined above, generally comprise a like number of amino acids sequences, although C-terminal truncations for the purpose of constructing soluble TNF-Rs will contain fewer amino acid sequences. In order to preserve the biological activity

of TNF-Rs, deletions and substitutions will preferably result in homologous or conservatively substituted sequences, meaning that a given residue is replaced by a biologically similar residue. Examples of conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other such conservative substitutions, for example, substitutions of entire regions having similar hydrophobicity characteristics, are well known. Moreover, particular amino acid differences between human, murine and other mammalian TNF-Rs is suggestive of additional conservative substitutions that may be made without altering the essential biological characteristics of TNF-R.

Subunits of TNF-R may be constructed by deleting terminal or internal residues or sequences. Particularly preferred sequences include those in which the transmembrane region and intracellular domain of TNF-R are deleted or substituted with hydrophilic residues to facilitate secretion of the receptor into the cell culture medium. The resulting protein is referred to as a soluble TNF-R molecule which retains its ability to bind TNF. A particularly preferred soluble TNF-R construct is TNF-RA235 (the sequence of amino acids 1-235 of FIG. 2A), which comprises the entire extracellular region of TNF-R, terminating with Asp²³⁵ immediately adjacent the transmembrane region. Additional amino acids may be deleted from the transmembrane region while retaining TNF binding activity. For example, huTNF-RA183 which comprises the sequence of amino acids 1-183 of FIG. 2A, and TNF-RA163 which comprises the sequence of amino acids 1-163 of FIG. 2A, retain the ability to bind TNF ligand as determined using the binding assays described below in Example 1. TNF-RA142, however, does not retain the ability to bind TNF ligand. This suggests that one or both of Cys¹⁵⁷ and Cys¹⁶³ is required for formation of an intramolecular disulfide bridge for the proper folding of TNF-R. Cys¹⁷⁸, which was deleted without any apparent adverse effect on the ability of the soluble TNF-R to bind TNF, does not appear to be essential for proper folding of TNF-R. Thus, any deletion C-terminal to Cys¹⁶³ would be expected to result in a biologically active soluble TNF-R. The present invention contemplates such soluble TNF-R constructs corresponding to all or part of the extracellular region of TNF-R terminating with any amino acid after Cys¹⁶³. Other C-terminal deletions, such as TNF-RA157, may be made as a matter of convenience by cutting TNF-R cDNA with appropriate restriction enzymes and, if necessary, reconstructing specific sequences with synthetic oligonucleotide linkers. The resulting soluble TNF-R constructs are then inserted and expressed in appropriate expression vectors and assayed for the ability to bind TNF, as described in Example 1. Biologically active soluble TNF-Rs resulting from such constructions are also contemplated to be within the scope of the present invention.

Mutations in nucleotide sequences constructed for expression of analog TNF-R must, of course, preserve the reading frame phase of the coding sequences and preferably will not create complementary regions that could hybridize to produce secondary mRNA structures such as loops or hairpins which would adversely affect translation of the receptor mRNA. Although a mutation site may be predetermined, it is not necessary

that the nature of the mutation per se be predetermined. For example, in order to select for optimum characteristics of mutants at a given site, random mutagenesis may be conducted at the target codon and the expressed TNF-R mutants screened for the desired activity.

Not all mutations in the nucleotide sequence which encodes TNF-R will be expressed in the final product, for example, nucleotide substitutions may be made to enhance expression, primarily to avoid secondary structure loops in the transcribed mRNA (see EPA 75,444A, incorporated herein by reference), or to provide codons that are more readily translated by the selected host, e.g., the well-known *E. coli* preference codons for *E. coli* expression.

Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (*Gene* 42:133, 1986); Bauer et al. (*Gene* 37:73, 1985); Craik (*BioTechniques*, Jan. 12-19, 1985); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); and U.S. Pat. Nos. 4,518,584 and 4,737,462 disclose suitable techniques, and are incorporated by reference herein.

Both monovalent forms and polyvalent forms of TNF-R are useful in the compositions and methods of this invention. Polyvalent forms possess multiple TNF-R binding sites for TNF ligand. For example, a bivalent soluble TNF-R may consist of two tandem repeats of amino acids 1-235 of FIG. 2A, separated by a linker region. Alternate polyvalent forms may also be constructed, for example, by chemically coupling TNF-R to any clinically acceptable carrier molecule, a polymer selected from the group consisting of Ficoll, polyethylene glycol or dextran using conventional coupling techniques. Alternatively, TNF-R may be chemically coupled to biotin, and the biotin-TNF-R conjugate then allowed to bind to avidin, resulting in tetravalent avidin/biotin/TNF-R molecules. TNF-R may also be covalently coupled to dinitrophenol (DNP) or trinitrophenol (TNP) and the resulting conjugate precipitated with anti-DNP or anti-TNP-IgM, to form decameric conjugates with a valency of 10 for TNF-R binding sites.

A recombinant chimeric antibody molecule may also be produced having TNF-R sequences substituted for the variable domains of either or both of the immunoglobulin molecule heavy and light chains and having unmodified constant region domains. For example, chimeric TNF-R/IgG₁ may be produced from two chimeric genes—a TNF-R/human κ light chain chimera (TNF-R/C _{κ}) and a TNF-R/human γ ₁ heavy chain chimera (TNF-R/C _{γ -1}). Following transcription and translation of the two chimeric genes, the gene products assemble into a single chimeric antibody molecule having TNF-R displayed bivalently. Such polyvalent forms of TNF-R may have enhanced binding affinity for TNF ligand. Additional details relating to the construction of such chimeric antibody molecules are disclosed in WO 89/09622 and EP 315062.

Expression of Recombinant TNF-R

The present invention provides recombinant expression vectors to amplify or express DNA encoding TNF-R. Recombinant expression vectors are replicable DNA constructs which have synthetic or cDNA-derived DNA fragments encoding mammalian TNF-R or bio-equivalent analogs operably linked to suitable transcriptional or translational regulatory elements derived from mammalian, microbial, viral or insect genes. A transcriptional unit generally comprises an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, transcriptional promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences, as described in detail below. Such regulatory elements may include an operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated. DNA regions are operably linked when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operably linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of secretory leaders, contiguous and in reading frame. Structural elements intended for use in yeast expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may optionally be subsequently cleaved from the expressed recombinant protein to provide a final product.

DNA sequences encoding mammalian TNF receptors which are to be expressed in a microorganism will preferably contain no introns that could prematurely terminate transcription of DNA into mRNA; however, premature termination of transcription may be desirable, for example, where it would result in mutants having advantageous C-terminal truncations, for example, deletion of a transmembrane region to yield a soluble receptor not bound to the cell membrane. Due to code degeneracy, there can be considerable variation in nucleotide sequences encoding the same amino acid sequence. Other embodiments include sequences capable of hybridizing to the sequences of the provided cDNA under moderately stringent conditions (50° C., 2× SSC) and other sequences hybridizing or degenerate to those which encode biologically active TNF receptor polypeptides.

Recombinant TNF-R DNA is expressed or amplified in a recombinant expression system comprising a substantially homogeneous monoculture of suitable host microorganisms, for example, bacteria such as *E. coli* or yeast such as *S. cerevisiae*, which have stably integrated (by transformation or transfection) a recombinant transcriptional unit into chromosomal DNA or carry the recombinant transcriptional unit as a component of a resident plasmid. Generally, cells constituting the sys-

tem are the progeny of a single ancestral transformant. Recombinant expression systems as defined herein will express heterologous protein upon induction of the regulatory elements linked to the DNA sequence or synthetic gene to be expressed.

Transformed host cells are cells which have been transformed or transfected with TNF-R vectors constructed using recombinant DNA techniques. Transformed host cells ordinarily express TNF-R, but host cells transformed for purposes of cloning or amplifying TNF-R DNA do not need to express TNF-R. Expressed TNF-R will be deposited in the cell membrane or secreted into the culture supernatant, depending on the TNF-R DNA selected. Suitable host cells for expression of mammalian TNF-R include prokaryotes, yeast or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Cell-free translation systems could also be employed to produce mammalian TNF-R using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described by Pouwels et al. (*Cloning Vectors: A Laboratory Manual*, Elsevier, N.Y., 1985), the relevant disclosure of which is hereby incorporated by reference.

Prokaryotic expression hosts may be used for expression of TNF-R that do not require extensive proteolytic and disulfide processing. Prokaryotic expression vectors generally comprise one or more phenotypic selectable markers, for example a gene encoding proteins conferring antibiotic resistance or supplying an autotrophic requirement, and an origin of replication recognized by the host to ensure amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

Useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, Wis., USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed. *E. coli* is typically transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species (Bolívar et al., *Gene* 2:95, 1977). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells.

Promoters commonly used in recombinant microbial expression vectors include the β -lactamase (penicillinase) and lactose promoter system (Chang et al., *Nature* 275:615, 1978; and Goeddel et al., *Nature* 281:544, 1979), the tryptophan (up) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, 1980; and EPA 36,776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful bacterial expression system employs the phage λ P_L promoter and cI857ts thermolabile repressor. Plasmid vectors available from the American Type Culture Collection which incorporate

derivatives of the λ P_L promoter include plasmid pHUB2, resident in *E. coli* strain JMB9 (ATCC 37092) and pPLc28, resident in *E. coli* RR1 (ATCC 53082).

Recombinant TNF-R proteins may also be expressed in yeast hosts, preferably from the *Saccharomyces* species, such as *S. cerevisiae*. Yeast of other genera, such as *Pichia* or *Kluyveromyces* may also be employed. Yeast vectors will generally contain an origin of replication from the 2 μ yeast plasmid or an autonomously replicating sequence (ARS), promoter, DNA encoding TNF-R, sequences for polyadenylation and transcription termination and a selection gene. Preferably, yeast vectors will include an origin of replication and selectable marker permitting transformation of both yeast and *E. coli*, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 or URA3 gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, and a promoter derived from a highly expressed yeast gene to induce transcription of the structural sequence downstream. The presence of the TRP1 or URA3 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan or uracil.

Suitable promoter sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149, 1968; and Holland et al., *Biochem.* 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triose-phosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPA 73,657.

Preferred yeast vectors can be assembled using DNA sequences from pUC18 for selection and replication in *E. coli* (Amp^r gene and origin of replication) and yeast DNA sequences including a glucose-repressible ADH2 promoter and α -factor secretion leader. The ADH2 promoter has been described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). The yeast α -factor leader, which directs secretion of heterologous proteins, can be inserted between the promoter and the structural gene to be expressed. See, e.g., Kurjan et al., *Cell* 30:933, 1982; and Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984. The leader sequence may be modified to contain, near its 3' end, one or more useful restriction sites to facilitate fusion of the leader sequence to foreign genes.

Suitable yeast transformation protocols are known to those of skill in the art; an exemplary technique is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929, 1978, selecting for Trp⁺ transformants in a selective medium consisting of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μ g/ml adenine and 20 μ g/ml uracil or URA⁺ transformants in medium consisting of 0.67% YNB, with amino acids and bases as described by Sherman et al., *Laboratory Course Manual for Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1986.

Host strains transformed by vectors comprising the ADH2 promoter may be grown for expression in a rich medium consisting of 1% yeast extract, 2% peptone, and 1% or 4% glucose supplemented with 80 μ g/ml

adenine and 80 μ g/ml uracil. Derepression of the ADH2 promoter occurs upon exhaustion of medium glucose. Crude yeast supernatants are harvested by filtration and held at 4° C. prior to further purification.

Various mammalian or insect cell culture systems are also advantageously employed to express recombinant protein. Expression of recombinant proteins in mammalian cells is particularly preferred because such proteins are generally correctly folded, appropriately modified and completely functional. Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells, described by Gluzman (*Cell* 23:175, 1981), and other cell lines capable of expressing an appropriate vector including, for example, L cells, C127, 3T3, Chinese hamster ovary (CHO), HeLa and BHK cell lines. Mammalian expression vectors may comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988).

The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells may be provided by viral sources. For example, commonly used promoters and enhancers are derived from Polyoma, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a heterologous DNA sequence. The early and late promoters are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., *Nature* 273:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the Hind 3 site toward the Bgl1 site located in the vital origin of replication is included. Further, mammalian genomic TNF-R promoter, control and/or signal sequences may be utilized, provided such control sequences are compatible with the host cell chosen. Additional details regarding the use of a mammalian high expression vector to produce a recombinant mammalian TNF receptor are provided in Examples 2 and 7 below. Exemplary vectors can be constructed as disclosed by Okayama and Berg (*Mol. Cell Biol.* 3:280, 1983).

A useful system for stable high level expression of mammalian receptor cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol.* 23:935, 1986).

In preferred aspects of the present invention, recombinant expression vectors comprising TNF-R cDNAs are stably integrated into a host cell's DNA. Elevated levels of expression product is achieved by selecting for cell lines having amplified numbers of vector DNA. Cell lines having amplified numbers of vector DNA are selected, for example, by transforming a host cell with a vector comprising a DNA sequence which encodes an enzyme which is inhibited by a known drug. The vector may also comprise a DNA sequence which encodes a desired protein. Alternatively, the host cell may be

co-transformed with a second vector which comprises the DNA sequence which encodes the desired protein. The transformed or co-transformed host cells are then cultured in increasing concentrations of the known drug, thereby selecting for drug-resistant cells. Such drug-resistant cells survive in increased concentrations of the toxic drug by over-production of the enzyme which is inhibited by the drug, frequently as a result of amplification of the gene encoding the enzyme. Where drug resistance is caused by an increase in the copy number of the vector DNA encoding the inhibitable enzyme, there is a concomitant co-amplification of the vector DNA encoding the desired protein (TNF-R) in the host cell's DNA.

A preferred system for such co-amplification uses the gene for dihydrofolate reductase (DHFR), which can be inhibited by the drug methotrexate (MTX). To achieve co-amplification, a host cell which lacks an active gene encoding DHFR is either transformed with a vector which comprises DNA sequence encoding DHFR and a desired protein, or is co-transformed with a vector comprising a DNA sequence encoding DHFR and a vector comprising a DNA sequence encoding the desired protein. The transformed or co-transformed host cells are cultured in media containing increasing levels of MTX, and those cells lines which survive are selected.

A particularly preferred co-amplification system uses the gene for glutamine synthetase (GS), which is responsible for the synthesis of glutamate and ammonia using the hydrolysis of ATP to ADP and phosphate to drive the reaction. GS is subject to inhibition by a variety of inhibitors, for example methionine sulfoximine (MSX). Thus, TNF-R can be expressed in high concentrations by co-amplifying cells transformed with a vector comprising the DNA sequence for GS and a desired protein, or co-transformed with a vector comprising a DNA sequence encoding GS and a vector comprising a DNA sequence encoding the desired protein, culturing the host cells in media containing increasing levels of MSX and selecting for surviving cells. The GS co-amplification system, appropriate recombinant expression vectors and cells lines, are described in the following PCT applications: WO 87/04462, WO 89/01036, WO 89/10404 and WO 86/05807.

Recombinant proteins are preferably expressed by co-amplification of DHFR or GS in a mammalian host cell, such as Chinese Hamster Ovary (CHO) cells, or alternatively in a murine myeloma cell line, such as SP2/0-Ag14 or NS0 or a rat myeloma cell line, such as YB2/3.0-Ag20, disclosed in PCT applications WO/89/10404 and WO 86/05807.

A preferred eukaryotic vector for expression of TNF-R DNA is disclosed below in Example 2. This vector, referred to as pCAV/NOT, was derived from the mammalian high expression vector pDC201 and contains regulatory sequences from SV40, adenovirus-2, and human cytomegalovirus.

Purification of Recombinant TNF-R

Purified mammalian TNF receptors or analogs are prepared by culturing suitable host/vector systems to express the recombinant translation products of the DNAs of the present invention, which are then purified from culture media or cell extracts.

For example, supernatants from systems which secrete recombinant protein into culture media can be first concentrated using a commercially available pro-

tein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a suitable purification matrix. For example, a suitable affinity matrix can comprise a TNF or lectin or antibody molecule bound to a suitable support. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred.

Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify a TNF-R composition. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

Recombinant protein produced in bacterial culture is usually isolated by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography steps. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of recombinant mammalian TNF-R can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Fermentation of yeast which express mammalian TNF-R as a secreted protein greatly simplifies purification. Secreted recombinant protein resulting from a large-scale fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog.* 296:171, 1984). This reference describes two sequential, reversed-phase HPLC steps for purification of recombinant human GM-CSF on a preparative HPLC column.

Human TNF-R synthesized in recombinant culture is characterized by the presence of non-human cell components, including proteins, in amounts and of a character which depend upon the purification steps taken to recover human TNF-R from the culture. These components ordinarily will be of yeast, prokaryotic or non-human higher eukaryotic origin and preferably are present in innocuous contaminant quantities, on the order of less than about 1 percent by weight. Further, recombinant cell culture enables the production of TNF-R free of proteins which may be normally associated with TNF-R as it is found in nature in its species of origin, e.g. in cells, cell exudates or body fluids.

Therapeutic Administration of Recombinant Soluble TNF-R

The present invention provides methods of using therapeutic compositions comprising an effective amount of soluble TNF-R proteins and a suitable diluent and carrier, and methods for suppressing TNF-dependent inflammatory responses in humans comprising administering an effective amount of soluble TNF-R protein.

For therapeutic use, purified soluble TNF-R protein is administered to a patient, preferably a human, for

treatment in a manner appropriate to the indication. Thus, for example, soluble TNF-R protein compositions can be administered by bolus injection, continuous infusion, sustained release from implants, or other suitable technique. Typically, a soluble TNF-R therapeutic agent will be administered in the form of a composition comprising purified protein in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the TNF-R with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents. Preferably, product is formulated as a lyophilizate using appropriate excipient solutions (e.g., sucrose) as diluents. Appropriate dosages can be determined in trials. The amount and frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the condition of the patient, and so forth.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

Example 1

Binding Assays

A. Radiolabeling of TNF α and TNF β .

Recombinant human TNF α , in the form of a fusion protein containing a hydrophilic octapeptide at the N-terminus, was expressed in yeast as a secreted protein and purified by affinity chromatography (Hopp et al., *Bio/Technology* 6:1204, 1988). Purified recombinant human TNF β was purchased from R & D Systems (Minneapolis, Minn.). Both proteins were radiolabeled using the commercially available solid phase agent, IODO-GEN (Pierce). In this procedure, 5 μ g of IODO-GEN were plated at the bottom of a 10 \times 75 mm glass tube and incubated for 20 minutes at 4 $^{\circ}$ C. with 75 μ l of 0.1M sodium phosphate, pH 7.4 and 20 μ l (2 mCi) Na 125 I. This solution was then transferred to a second glass tube containing 5 μ g TNF α (or TNF β) in 45 μ l PBS for 20 minutes at 4 $^{\circ}$ C. The reaction mixture was fractionated by gel filtration on a 2 ml bed volume of Sephadex G-25 (Sigma) equilibrated in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2.5% (w/v) bovine serum albumin (BSA), 0.2% (w/v) sodium azide and 20 mM Hepes pH 7.4 (binding medium). The final pool of 125 I-TNF was diluted to a working stock solution of 1×10^{-7} M in binding medium and stored for up to one month at 4 $^{\circ}$ C. without detectable loss of receptor binding activity. The specific activity is routinely 1×10^6 cpm/mmol TNF.

B. Binding to Intact Cells.

Binding assays with intact cells were performed by two methods. In the first method, cells were first grown either in suspension (e.g., U 937) or by adherence on tissue culture plates (e.g., WI26-VA4, COS cells expressing the recombinant TNF receptor). Adherent cells were subsequently removed by treatment with 5 mM EDTA treatment for ten minutes at 37 degrees centigrade. Binding assays were then performed by a phthalate oil separation method (Dower et al., *J. Immunol.* 132:751, 1984) essentially as described by

Park et al. (*J. Biol. Chem.* 261:4177, 1986). Non-specific binding of 125 I-TNF was measured in the presence of a 200-fold or greater molar excess of unlabeled TNF. Sodium azide (0.2%) was included in a binding assay to inhibit internalization of 125 I-TNF by cells. In the second method, COS cells transfected with the TNF-R-containing plasmid, and expressing TNF receptors on the surface, were tested for the ability to bind 125 I-TNF by the plate binding assay described by Sims et al. (*Science* 241:585, 1988).

C. Solid Phase Binding Assays.

The ability of TNF-R to be stably adsorbed to nitrocellulose from detergent extracts of human cells yet retain TNF-binding activity provided a means of detecting TNF-R. Cell extracts were prepared by mixing a cell pellet with a 2 \times volume of PBS containing 1% Triton X-100 and a cocktail of protease inhibitors (2 mM phenylmethyl sulfonyl fluoride, 10 μ M pepstatin, 10 μ M leupeptin, 2 mM o-phenanthroline and 2 mM EGTA) by vigorous vortexing. The mixture was incubated on ice for 30 minutes after which it was centrifuged at 12,000 \times g for 15 minutes at 8 $^{\circ}$ C. to remove nuclei and other debris. Two microliter aliquots of cell extracts were placed on dry BA85/21 nitrocellulose membranes (Schleicher and Schuell, Keene, N.H.) and allowed to dry. The membranes were incubated in tissue culture dishes for 30 minutes in Tris (0.05M) buffered saline (0.15M) pH 7.5 containing 3% w/v BSA to block nonspecific binding sites. The membrane was then covered with 5×10^{-11} M 125 I-TNF in PBS+3% BSA and incubated for 2 hr at 4 $^{\circ}$ C. with shaking. At the end of this time, the membranes were washed 3 times in PBS, dried and placed on Kodak X-Omat AR film for 18 hr at -70 $^{\circ}$ C.

Example 2

Isolation of Human TNF-R cDNA by Direct Expression of Active Protein in COS-7 Cells

Various human cell lines were screened for expression of TNF-R based on their ability to bind 125 I-labeled TNF. The human fibroblast cell line WI-26 VA4 was found to express a reasonable number of receptor per cell. Equilibrium binding studies showed that the cell line exhibited biphasic binding of 125 I-TNF with approximately 4,000 high affinity sites ($K_d = 1 \times 10^{10}$ M $^{-1}$) and 15,00 low affinity sites ($K_d = 1 \times 10^8$ M $^{-1}$) per cell.

An unsized cDNA library was constructed by reverse transcription of polyadenylated mRNA isolated from total RNA extracted from human fibroblast WI-26 VA4 cells grown in the presence of pokeweed mitogen using standard techniques (Gubler, et al., *Gene* 25:263, 1983; Ausubel et al., eds., *Current Protocols in Molecular Biology*, Vol. 1, 1987). The cells were harvested by lysing the cells in a guanidine hydrochloride solution and total RNA isolated as previously described (March et al., *Nature* 315:641, 1985).

Poly A $^{+}$ RNA was isolated by oligo dT cellulose chromatography and double-stranded cDNA was prepared by a method similar to that of Gubler and Hoffman (*Gene* 25:263, 1983). Briefly, the poly A $^{+}$ RNA was converted to an RNA-cDNA hybrid by reverse transcriptase using oligo dT as a primer. The RNA-cDNA hybrid was then converted into double-stranded cDNA using RNAase H in combination with DNA polymerase I. The resulting double stranded cDNA was blunt-ended with T4 DNA polymerase. To the blunt-ended

cDNA is added EcoRI linker-adapters (having internal NotI sites) which were phosphorylated on only one end (Invitrogen). The linker-adapted cDNA was treated with T4 polynucleotide kinase to phosphorylate the 5' overhanging region of the linker-adapter and unligated linkers were removed by running the cDNA over a Sepharose CL4B column. The linker-adapted cDNA was ligated to an equimolar concentration of EcoRI cut and dephosphorylated arms of bacteriophage λ gt10 (Huynh et al., *DNA Cloning: A Practical Approach*, Glover, ed., IRL Press, pp. 49-78). The ligated DNA was packaged into phage particles using a commercially available kit to generate a library of recombinants (Stratagene Cloning Systems, San Diego, Calif., USA). Recombinants were further amplified by plating phage on a bacterial lawn of *E. coli* strain c600(hf1-).

Phage DNA was purified from the resulting λ gt10 cDNA library and the cDNA inserts excised by digestion with the restriction enzyme NotI. Following electrophoresis of the digest through an agarose gel, cDNAs greater than 2,000 bp were isolated.

The resulting cDNAs were ligated into the eukaryotic expression vector pCAV/NOT, which was designed to express cDNA sequences inserted at its multiple cloning site when transfected into mammalian cells. pCAV/NOT was assembled from pDC201 (a derivative of pMLSV, previously described by Cosman et al., *Nature* 312: 768, 1984), SV40 and cytomegalovirus DNA and comprises, in sequence with the direction of transcription from the origin of replication: (1) SV40 sequences from coordinates 5171-270 including the origin of replication, enhancer sequences and early and late promoters; (2) cytomegalovirus sequences including the promoter and enhancer regions (nucleotides 671 to +63 from the sequence published by Boechart et al. (*Cell* 41:521, 1985); (3) adenovirus-2 sequences containing the first exon and part of the intron between the first and second exons of the tripartite leader, the second exon and part of the third exon of the tripartite leader and a multiple cloning site (MCS) containing sites for XhoI, KpnI, SmaI, NotI and BglI; (4) SV40 sequences from coordinates 4127-4100 and 2770-2533 that include the polyadenylation and termination signals for early transcription; (5) sequences derived from pBR322 and virus-associated sequences VAI and VAII of pDC201, with adenovirus sequences 10532-11156 containing the VAI and VAII genes, followed by pBR322 sequences from 4363-2486 and 1094-375 containing the ampicillin resistance gene and origin of replication.

The resulting WI-26 VA4 cDNA library in pCAV/NOT was used to transform *E. coli* strain DH5 α , and recombinants were plated to provide approximately 800 colonies per plate and sufficient plates to provide approximately 50,000 total colonies per screen. Colonies were scraped from each plate, pooled, and plasmid DNA prepared from each pool. The pooled DNA was then used to transfect a sub-confluent layer of monkey COS-7 cells using DEAE-dextran followed by chloroquine treatment, as described by Luthman et al. (*Nucl. Acids Res.* 11:1295, 1983) and McCutchan et al. (*J. Natl. Cancer Inst.* 41:351, 1986). The cells were then grown in culture for three days to permit transient expression of the inserted sequences. After three days, cell culture supernatants were discarded and the cell monolayers in each plate assayed for TNF binding as follows. Three ml of binding medium containing 1.2×10^{-11} M 125 I-labeled FLAG®-TNF was added to each plate and the plates incubated at 4° C. for 120 minutes. This medium

was then discarded, and each plate was washed once with cold binding medium (containing no labeled TNF) and twice with cold PBS. The edges of each plate were then broken off, leaving a flat disk which was contacted with X-ray film for 72 hours at -70° C. using an intensifying screen. TNF binding activity was visualized on the exposed films as a dark focus against a relatively uniform background.

After approximately 240,000 recombinants from the library had been screened in this manner, one transfectant pool was observed to provide TNF binding foci which were clearly apparent against the background exposure.

A frozen stock of bacteria from the positive pool was then used to obtain plates of approximately 150 colonies. Replicas of these plates were made on nitrocellulose filters, and the plates were then scraped and plasmid DNA prepared and transfected as described above to identify a positive plate. Bacteria from individual colonies from the nitrocellulose replica of this plate were grown in 0.2 ml cultures, which were used to obtain plasmid DNA, which was transfected into COS-7 cells as described above. In this manner, a single clone, clone 1, was isolated which was capable of inducing expression of human TNF-R in COS cells. The expression vector pCAV/NOT containing the TNF-R cDNA clone 1 has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, USA (Accession No. 68088) under the name pCAV/NOT-TNF-R.

Example 3

Construction of cDNAs Encoding Soluble huTNF-RA235

A cDNA encoding a soluble huTNF-RA235 (having the sequence of amino acids -22-235 of FIG. 2A) was constructed by excising an 840 bp fragment from pCAV/NOT-TNF-R with the restriction enzymes NotI and Pvu2. NotI cuts at the multiple cloning site of pCAV/NOT-TNF-R and Pvu2 cuts within the TNF-R coding region 20 nucleotides 5' of the transmembrane region. In order to reconstruct the 3' end of the TNF-R sequences, two oligonucleotides were synthesized and annealed to create the following oligonucleotide linker:

```

Pvu2                               BamHI Bgl2
CTGAAGGGAGCACTGGCGACTAAGGATCCA
GACTTCCCTCGTGACCGCTGATTCTAGGTCTAG
AlaGluGlySerThrGlyAspEnd

```

This oligonucleotide linker has terminal Pvu2 and Bgl2 restriction sites, regenerates 20 nucleotides of the TNF-R, followed by a termination codon (underlined) and a BamHI restriction site (for convenience in isolating the entire soluble TNF-R by NotI/BamHI digestion). This oligonucleotide was then ligated with the 840 bp NotI/-Pvu2 TNF-R insert into Bgl2/NotI cut pCAV/NOT to yield psolhuTNF-RA235/CAVNOT, which was transfected into COS-7 cells as described above. This expression vector induced expression and secretion by the host cell of soluble human TNF-R (having the sequence of amino acids 1-235 of FIG. 2A) which was capable of binding TNF.

Example 4

Construction of cDNAs Encoding Soluble huTNF-RA185

A cDNA for expressing a soluble huTNF-RA185 (having the sequence of amino acids 1-185 of FIG. 2A) was constructed by excising a 640 bp fragment from pCAV/NOT-TNF-R with the restriction enzymes Not1 and Bgl2. Not1 cuts at the multiple cloning site of pCAV/NO-TNF-R and Bgl2 cuts within the TNF-R coding region at nucleotide 637, which is 237 nucleo-

tides 5' of the transmembrane region. The following oligonucleotide linkers were synthesized:

Bgl2
5'-GATCTGTAAACGTGGTGGCCATCCCTGGGAATGCAAGCATGGATGC-3'
ACATTGCACCAACCGGTAGGGACCCTTACGTTCCG
IleCysAsnValValAlaIleProGlyAsnAlaSerMetAspAla

Not1
5'-AGTCTGCACGTCCACGTCCCCACCCGGTGAGC-3'
TACCTACGTCAGACGTGCAGGTGCAGGGGGTGGGCCACTCGCCGG
ValCysThrSerThrSerProThrArgEnd

The above oligonucleotide linkers reconstruct the 3' end of the receptor molecule up to nucleotide 708, followed by a termination codon (underlined). These oligonucleotides were then ligated with the 640 bp Not1 TNF-R insert into Not1 cut pCAV/NOT to yield the expression vector psolTNFRA185/CAVNOT, which was transfected into COS-7 cells as described above. This expression vector induced expression of soluble human TNF-R (having the sequence of amino acids 1-163 of FIG. 2A) which was capable of binding TNF.

Example 5

Construction of cDNAs Encoding Soluble huTNF-RA163

A cDNA for expressing a soluble huTNF-RA163 (having the sequence of amino acids 1-163 of FIG. 2A) was constructed by excising a 640 bp fragment from pCAV/NOT-TNF-R with the restriction enzymes Not1 and Bgl2 as described in Example 4. The following oligonucleotide linkers were synthesized:

Bgl2 Not1
5'-GATCTGTGAGC-3'
ACAACCTCGCCGG
IleCysEnd

This above oligonucleotide linker reconstructs the 3' end of the receptor molecule up to nucleotide 642 (amino acid 163), followed by a termination codon (underlined). This oligonucleotide was then ligated with the 640 bp Not1 TNF-R insert into Not1 cut pCAV/NOT to yield the expression vector psolTNFRA163/CAVNOT, which was transfected into COS-7 cells as described above. This expression vector induced expression of soluble human TNF-R (having the sequence of amino acids 1-163 of FIG. 2A) which was capable of binding TNF in the binding assay described in Example 1.

Example 6

Construction of cDNAs Encoding Soluble huTNF-RA142

A cDNA for expressing a soluble huTNF-RA142 (having the sequence of amino acids 1-142 of FIG. 2A) was constructed by excising a 550 bp fragment from pCAV/NOT-TNF-R with the restriction enzymes Not1 and Al wN1. Al wN1 cuts within the TNF-R coding region at nucleotide 549. The following oligonucleotide linker was synthesized:

Bgl2 Not1
5'-CTGAAACATCAGACGTGGTGTGCAAGCCCTGTTAAA-3'
CTTGACTTGTAGTCTGCACCACACGTTCCGGACAATTCTAGA
End

This above oligonucleotide linker reconstructs the 3' end of the receptor molecule up to nucleotide 579

(amino acid 142), followed by a termination codon (underlined). This oligonucleotide was then ligated with the 550 bp Not1/Al wN1 TNF-R insert into Not1/Bgl2 cut pCAV/NOT to yield the expression vector psolTNFRA142/CAVNOT, which was transfected into COS-7 cells as described above. This expression vector did not induced expression of soluble human TNF-R which was capable of binding TNF. It is believed that this particular construct failed to express biologically active TNF-R because one or more essential cysteine residue (e.g., Cys¹⁵⁷ or Cys¹⁶³) required for intramolecular bonding (for formation of the proper tertiary structure of the TNF-R molecule) was eliminated.

Example 7

Expression of Soluble TNF Receptors in CHO Cells

Soluble TNF receptor was expressed in Chinese Hamster Ovary (CHO) cells using the glutamine-synthetase (GS) gene amplification system, substantially as described in PCT patent application Nos. WO87/04462 and WO89/01036. Briefly, CHO cells are transfected with an expression vector containing genes for both TNF-R and GS. CHO cells are selected for GS gene expression based on the ability of the transfected DNA to confer resistance to low levels of methionine sulfoximine (MSX). GS sequence amplification events in such cells are selected using elevated MSX concentrations. In this way, contiguous TNF-R sequences are also amplified and enhanced TNF-R expression is achieved.

The vector used in the GS expression system was psolTNFR/P6/PSVLGS, which was constructed as follows. First, the vector pSVLGS.1 (described in PCT Application Nos. WO87/04462 and WO89/01036, and available from Celltech, Ltd., Berkshire, UK) was cut with the BamHI restriction enzyme and dephosphorylated with calf intestinal alkaline phosphatase

(CIAP) to prevent the vector from religating to itself. The BamHI cut pSVLGS.1 fragment was then ligated to a 2.4 kb BarnI to BglII fragment of pEE6hCMV (described in PCT Application No. WO89/01036, also available from Celltech) which was cut with BglII, BamHI and FspI to avoid two fragments of similar size, to yield an 11.2 kb vector designated p6/PSVLGS.1. pSVLGS.1 contains the glutamine synthetase selectable marker gene under control of the SV40 later promoter. The BamHI to BglII fragment of pEE6hCMV contains the human cytomegalovirus major immediate early promoter (hCMV), a polylinker, and the SV40 early polyadenylation signal. The coding sequences for soluble TNF-R were added to p6/PSVLGS.1 by excising a NotI to BamHI fragment from the expression vector psoITNFR/CAVNOT (made according to Example 3 above), blunt ending with Klenow and ligating with SmaI cut dephosphorylated p6/PSVLGS.1, thereby placing the soITNFR coding sequences under the control of the hCMV promoter. This resulted in a single plasmid vector in which the SV40/GS and hCMB/soITNFR transcription units are transcribed in opposite directions. This vector was designated psoITNFR/P6/PSVLGS.

psoITNFR/P6/PSVLGS was used to transfect CHO-K1 cells (available from ATCC, Rockville, Md., under accession number CCL 61) as follows. A monolayer of CHO-K1 cells were grown to subconfluency in Minimum Essential Medium (MEM) 10X (Gibco: 330-1581AJ) without glutamine and supplemented with 10% dialysed fetal bovine serum (Gibco: 220-6300AJ), 1 mM sodium pyruvate (Sigma), MEM non-essential

amino acids (Gibco: 320-1140AG), 500 μ M asparagine and glutamate (Sigma) and nucleosides (30 μ M adenosine, guanosine, cytidine and uridine and 10 μ M thymidine) (Sigma).

Approximately 1×10^6 cells per 10 cm petri dish were transfected with 10 μ g of psoITNFR/P6/PSVLGS by standard calcium phosphate precipitation, substantially as described by Graham & van der Eb, *Virology* 52:456 (1983). Cells were subjected to glycerol shock (15% glycerol in serum-free culture medium for approximately 1.5 minutes) approximately 4 hours after transfection, substantially as described by Frost & Williams, *Virology* 91:39 (1978), and then washed with serum-free medium. One day later, transfected cells were fed with fresh selective medium containing MSX at a final concentration of 25 μ M. Colonies of MSX-resistant surviving cells were visible within 3-4 weeks. Surviving colonies were transferred to 24-well plates and allowed to grow to confluency in selective medium. Conditioned medium from confluent wells were then assayed for soluble TNF-R activity using the binding assay described in Example 1 above. These assays indicated that the colonies expressed biologically active soluble TNF-R.

In order to select for GS gene amplification, several MSX-resistant cell lines are transfected with

psoITNFR/P6/PSVLGS and grown in various concentrations of MSX. For each cell line, approximately 1×10^6 cells are plated in gradually increasing concentrations of 100 μ M, 250 μ M, 500 μ M and 1 mM MSX and incubated for 10-14 days. After 12 days, colonies resistant to the higher levels of MSX appear. The surviving colonies are assayed for TNF-R activity using the binding assay described above in Example 1. Each of these highly resistant cell lines contains cells which arise from multiple independent amplification events. From these cell lines, one or more of the most highly resistant cell lines are isolated. The amplified cells with high production rates are then cloned by limiting dilution cloning. Mass cell cultures of the transfectants secrete active soluble TNF-R.

Example 8

Expression of Soluble Human TNF-R in Yeast

Soluble human TNF-R was expressed in yeast with the expression vector pIXY432, which was derived from the yeast expression vector pIXY120 and plasmid pYEP352. pIXY120 is identical to pYaHuGM (ATCC 53157), except that it contains no cDNA insert and includes a polylinker/multiple cloning site with a NcoI restriction site.

A DNA fragment encoding TNF receptor and suitable for cloning into the yeast expression vector pIXY120 was first generated by polymerase chain reaction (PCR) amplification of the extracellular portion of the full length receptor from pCAV/NOT-TNF-R (ATCC 68088). The following primers were used in this PCR amplification:

```

5' End Primer
5'-TTCCGGTACCTTTGGATAAAAGAGACTACAAGGAC
Asp718----->ProLeuAspLysArgAspTyrLysAsp
GACGATGACAAGTTGCCGCCAGGTGGCATTTCATA-3'
AspAspAspLys<-----TNF-R----->

3' End Primer (antisense)
5'-CCCGGGATCCTTAGTCGCCAGTGCTCCCTTCAGCTGGG-3'
BamHI>End<-----TNF-R----->

```

The 5' end oligonucleotide primer used in the amplification included an Asp718 restriction site at its 5' end, followed by nucleotides encoding the 3' end of the yeast α -factor leader sequence (Pro-Leu-Asp-Lys-Arg) and those encoding the 8 amino acids of the FLAG® peptide (AspTyrLysAspAspAspAspLys) fused to sequence encoding the 5' end of the mature receptor. The FLAG® peptide (Hopp et al., *Bio/Technology* 6:1204, 1988) is a highly antigenic sequence which reversibly binds the monoclonal antibody M1 (ATCC HB 9259). The oligonucleotide used to generate the 3' end of the PCR-derived fragment is the antisense strand of DNA encoding sequences which terminate the open reading frame of the receptor after nucleotide 704 of the mature coding region (following the Asp residue preceding the transmembrane domain) by introducing a TAA stop codon (underlined). The stop codon is then followed by a BamHI restriction site. The DNA sequences encoding TNF-R are then amplified by PCR, substantially as described by Innis et al., eds., *PCR Protocols: A Guide to Methods and Applications* (Academic Press, 1990).

The PCR-derived DNA fragment encoding soluble human TNF-R was subcloned into the yeast expression vector pIXY120 by digesting the PCR-derived DNA

fragment with BamH1 and Asp718 restriction enzymes, digesting pIXY120 with BamH1 and Asp718, and ligating the PCR fragment into the cut vector in vitro with T4 DNA ligase. The resulting construction (pIXY424) fused the open reading frame of the FLAG®-soluble TNF receptor in-frame to the complete α -factor leader sequence and placed expression in yeast under the aegis of the regulated yeast alcohol dehydrogenase (ADH2) promoter. Identity of the nucleotide sequence of the soluble TNF receptor carded in pIXY424 with those in cDNA clone 1 were verified by DNA sequencing using the dideoxynucleotide chain termination method. pIXY424 was then transformed into *E. coli* strain RR1.

Soluble human TNF receptor was also expressed and secreted in yeast in a second vector. This second vector was generated by recovering the pIXY424 plasmid from *E. coli* and digesting with EcoR1 and BamH1 restriction enzymes to isolate the fragment spanning the region encoding the ADH2 promoter, the α -factor leader, the FLAG®-soluble TNF receptor and the stop codon. This fragment was ligated in vitro into EcoR1 and BamH1 cut plasmid pYEP352 (Hill et al., *Yeast* 2:163 (1986)), to yield the expression plasmid pIXY432, which was transformed into *E. coli* strain RR1.

To assess secretion of the soluble human TNF receptor from yeast, pIXY424 was purified and introduced into a diploid yeast strain of *S. cerevisiae* (XV2181) by electroporation and selection for acquisition of the plasmid-borne yeast TRP1⁺ gene on media lacking tryptophan. To assess secretion of the receptor directed by pIXY432, the plasmid was introduced into the yeast strain PB149-6b by electroporation followed by selection for the plasmid-borne URA3⁺ gene with growth on media lacking uracil. Overnight cultures were grown at 30° C. in the appropriate selective media. The PB149-6b/pIXY432 transformants were diluted into YEP-1% glucose media and grown at 30° C. for 38–40 hours. Supernatants were prepared by removal of cells by centrifugation, and filtration of supernatants through 0.45 μ filters.

The level of secreted receptor in the supernatants was determined by immuno-dot blot. Briefly, 1 μ l of supernatants, and dilutions of the supernatants, were spotted onto nitrocellulose filters and allowed to dry. After blocking non-specific protein binding with a 3% BSA solution, the filters were incubated with diluted M1 anti-FLAG® antibody, excess antibody was removed by washing and then dilutions of horseradish peroxidase conjugated anti-mouse IgG antibodies were incubated with the filters. After removal of excess secondary antibodies, peroxidase substrates were added and color development was allowed to proceed for approximately 10 minutes prior to removal of the substrate solution.

The anti-FLAG® reactive material found in the supernatants demonstrated that significant levels of receptor were secreted by both expression systems. Comparisons demonstrated that the pIXY432 system secreted approximately 8–16 times more soluble human TNF receptor than the pIXY424 system. The supernatants were assayed for soluble TNF-R activity, as described in Example 1, by their ability to bind ¹²⁵I-TNF α and block TNF α binding. The pIXY432 supernatants were found to contain significant levels of active soluble TNF-R.

Example 9

Isolation of Murine TNF-R cDNAs

Murine TNF-R cDNAs were isolated from a cDNA library made from murine 7B9 cells, an antigen-dependent helper T cell line derived from C57BL/6 mice, by cross-species hybridization with a human TNF-R probe. The cDNA library was constructed in λ ZAP (Stratagene, San Diego), substantially as described above in Example 2, by isolating polyadenylated RNA from the 7B9 cells.

A double-stranded human TNF-R cDNA probe was produced by excising an approximately 3.5 kb NotI fragment of the human TNF-R clone 1 and ³²P-labeling the cDNA using random primers (Boehringer-Mannheim).

The murine cDNA library was amplified once and a total of 900,000 plaques were screened, substantially as described in Example 2, with the human TNF-R cDNA probe. Approximately 21 positive plaques were purified, and the Bluescript plasmids containing EcoR1-linked inserts were excised (Stratagene, San Diego). Nucleic acid sequencing of a portion of murine TNF-R clone 11 indicated that the coding sequence of the murine TNF-R was approximately 88% homologous to the corresponding nucleotide sequence of human TNF-R. A partial nucleotide sequence of murine TNF-R cDNA clone 11 is set forth in FIGS. 3A–3B.

Example 10

Preparation of Monoclonal Antibodies to TNF-R

Preparations of purified recombinant TNF-R, for example, human TNF-R, or transfected COS cells expressing high levels of TNF-R are employed to generate monoclonal antibodies against TNF-R using conventional techniques, for example, those disclosed in U.S. Pat. No. 4,411,993. Such antibodies are likely to be useful in interfering with TNF binding to TNF receptors, for example, in ameliorating toxic or other undesired effects of TNF, or as components of diagnostic or research assays for TNF or soluble TNF receptor.

To immunize mice, TNF-R immunogen is emulsified in complete Freund's adjuvant and injected in amounts ranging from 10–100 μ g subcutaneously into Balb/c mice. Ten to twelve days later, the immunized animals are boosted with additional immunogen emulsified in incomplete Freund's adjuvant and periodically boosted thereafter on a weekly to biweekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision for testing by dot-blot assay (antibody sandwich) or ELISA (enzyme-linked immunosorbent assay). Other assay procedures are also suitable. Following detection of an appropriate antibody titer, positive animals are given an intravenous injection of antigen in saline. Three to four days later, the animals are sacrificed, splenocytes harvested, and fused to the murine myeloma cell line NS1. Hybridoma cell lines generated by this procedure are plated in multiple microtiter plates in a HAT selective medium (hypoxanthine, aminopterin, and thymidine) to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

Hybridoma clones thus generated can be screened by ELISA for reactivity with TNF-R, for example, by adaptations of the techniques disclosed by Engvall et al., *Immunochem.* 8:871 (1971) and in U.S. Pat. No. 4,703,004. Positive clones are then injected into the

peritoneal cavities of syngeneic Balb/c mice to produce ascites containing high concentrations (>1 mg/ml) of anti-TNF-R monoclonal antibody. The resulting monoclonal antibody can be purified by ammonium sulfate precipitation followed by gel exclusion chromatography, and/or affinity chromatography based on binding of antibody to Protein A of *Staphylococcus aureus*.

We claim:

1. An isolated DNA sequence consisting of a DNA sequence encoding only the amino acid sequence selected from the group consisting of amino acids 1-235 of FIG. 2A and amino acids 1-233 of FIG. 3A.

2. An isolated DNA sequence according to claim 1 which encodes a soluble human TNF-R.

3. An isolated DNA sequence consisting of a DNA sequence encoding only the amino acid sequence selected from the group consisting of amino acids 1 through x of FIG. 2A, wherein x is amino acid 163-235 of FIG. 2A.

4. An isolated DNA sequence according to claim 1 which encodes a soluble human TNF-R comprising the sequence of amino acids 1-235 of FIG. 2A.

5. An isolated DNA sequence according to claim 1 which encodes a soluble human TNF-R comprising the sequence of amino acids 1-185 of FIG. 2A.

6. An isolated DNA sequence consisting of a DNA sequence encoding only the amino acid sequence 1-163 of FIG. 2A.

7. An isolated DNA sequence according to claim 1 encoding the TNF-R polypeptide expressed by pCAV/NOT-TNF-R (ATCC 68088).

8. A recombinant expression vector comprising a DNA sequence according to claim 1.

9. A recombinant expression vector comprising a DNA sequence according to claim 2.

10. A recombinant expression vector comprising a DNA sequence according to claim 3.

11. A recombinant expression vector comprising a DNA sequence according to claim 4.

12. A recombinant expression vector comprising a DNA sequence according to claim 5.

13. A recombinant expression vector comprising a DNA sequence according to claim 6.

14. A recombinant expression vector comprising a DNA sequence according to claim 7.

15. A host cell transformed or transfected with the vector according to claim 9.

16. A host cell transformed or transfected with the vector according to claim 10.

17. A host cell transformed or transfected with the vector according to claim 11.

18. A host cell transformed or transfected with the vector according to claim 12.

19. A host cell transformed or transfected with the vector according to claim 13.

20. A host cell transformed or transfected with the vector according to claim 14.

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FUNDAMENTAL IMMUNOLOGY

SECOND EDITION

Editor

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Functions and Mechanisms of Lysis Induced by Cytotoxic T Lymphocytes and Natural Killer Cells

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It is quite fascinating that while metabolically and structurally different cells can live together harmoniously, certain specialized cells are also capable of damaging and even killing neighboring cells. Killing of one cell type by another through contact interaction constitutes a major effector arm of self-defense of the immune system. The

major immunologically relevant cytocidal cells other than macrophages are cytolytic T lymphocytes (CTLs), natural killer (NK) cells, and lymphokine-activated killer (LAK) cells. This chapter deals with cellular and molecular mechanisms involved in this wondrous mechanism of natural immunity, which assiduously protects the body

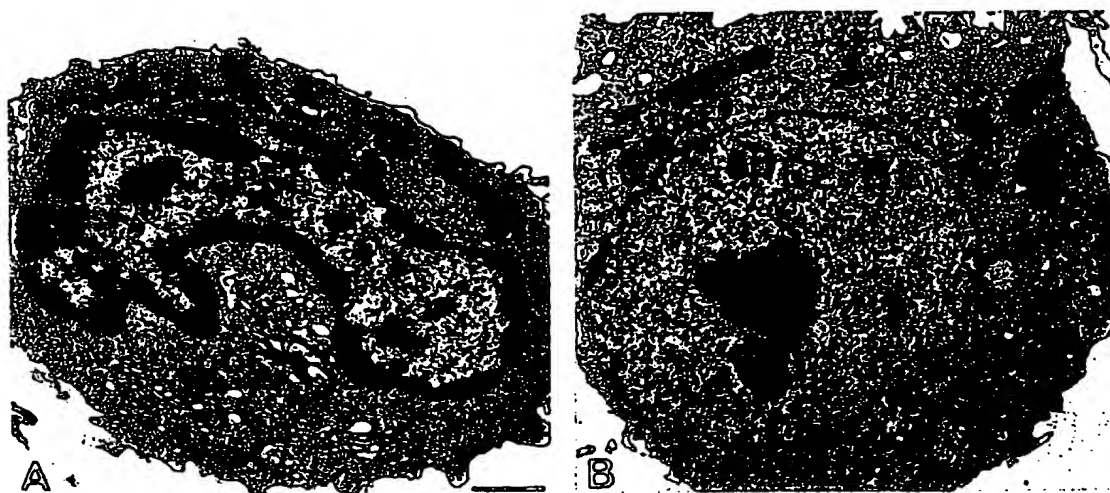


FIG. 1. Electron micrographs of nongranulated and granulated CTLs. The nongranulated CTL (A) are BALB/c anti-EL4 alloreactive peritoneal exudate cytolytic lymphocytes (PELs) (Berke et al., ref. 17). The PEL-blast (PEL-BL) (B) is derived from PELs cultured in the presence of Interleukin 2 (IL-2) (Berke and Rosen, ref. 88). Note the presence of osmiophilic granules (gr) in the PEL-blast and their absence in the PELs. gol, Golgi apparatus; mit, mitochondria. Bar represents 621 nm for A and 1960 nm for B. Electron microscopy by D. Rosen.

against viral, certain bacterial, and perhaps even cancerous diseases. On the negative side, however, killer lymphocytes form the primary obstacle to be overcome before tissue and organ transplants can be spared from rejection and before tissue damage in autoimmune diseases can be prevented.

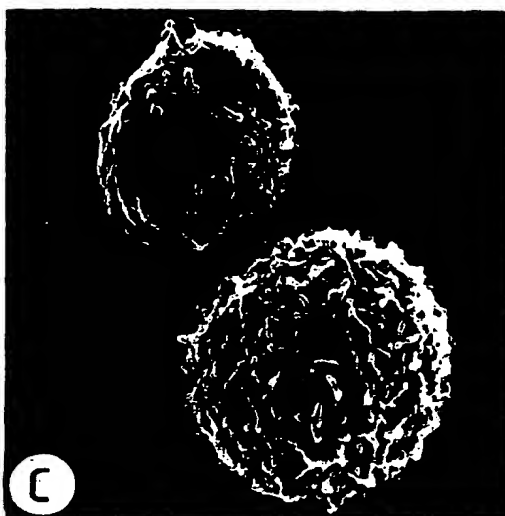
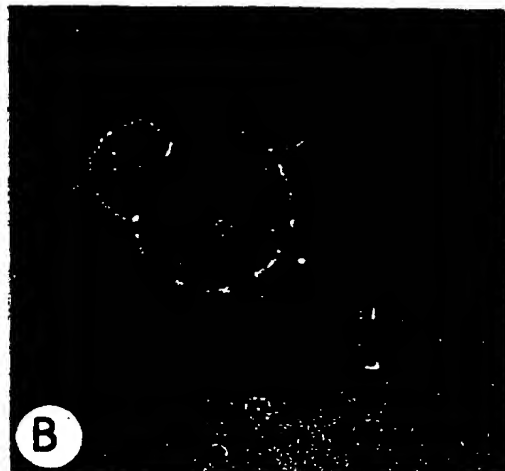
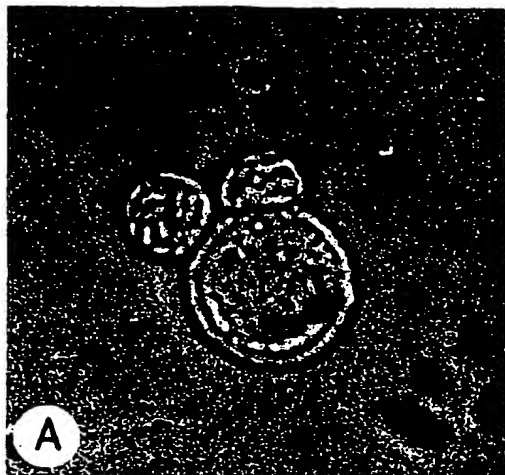
A major part of this chapter is devoted to the mechanism(s) whereby lymphocytes recognize and kill other cells, the subject of many previous investigations (reviews in refs. 1-4) since Govaert's original observation of cytotoxic lymphocytes in kidney allograft rejection. This topic is preceded by an introduction to the generation and classification of cytotoxic cells (reviews in refs. 5-8) and is followed by an assessment of their activities. We also discuss the function of cytotoxic cells in graft rejection, virus immunity, cancer immunotherapy, and the induction of tissue damage in autoimmunity (reviews in refs. 9-11).

CYTOTOXIC CELLS: THEIR GENERATION, RECEPTORS, AND MARKERS

Although predicted earlier, the discovery of specifically reactive cytotoxic lymphocytes must be credited to Govaert (12). The demonstration that sensitized cytotoxic

lymphocytes express the Thy-1 (formerly termed θ) antigen (Ag) on their cell surface resulted in their classification as cytotoxic T lymphocytes (CTLs) (13). Subsets of T lymphocytes were also discovered later. It is now known that the CD4 and CD8 cell surface molecules (L3T4 and Lyt2, respectively, in the mouse) are expressed on two mutually exclusive subsets of mature T lymphocytes. Most T helper cells (T_H) express CD4, whereas most cytotoxic/suppressor T cells (T_{CS}) express CD8. This correlation, however, appears not to be strict, as cytotoxic CD4⁺ T lymphocytes have been described (14), and cytotoxic CD4⁺ effectors may play a role in down regulating immune responses by killing autologous, antigen-presenting cells (B cells, macrophages). CD4⁺ cells always recognize antigen plus class II MHC molecules (even when they kill) and CD8⁺ cells recognize antigen plus class I (even when they do not kill). Some CTLs and most NK cells appear as large granular lymphocytes (LGLs) while mature *in vivo* primed CTLs are small to medium sized (8 to 12 μ m), nongranular lymphocytes. Transformation from the latter to the former type (Fig. 1) can be induced by interleukin 2 (IL-2) and probably by IL-4. Three distinct types of cytotoxic lymphocytes have been defined (15). The first are Ag-specific CTLs restricted by class I major histocompatibility complex (MHC) molecules that recognize the target through an idiotype T cell receptor (Ti) associated with another cell

FIG. 2. CTL-target cell conjugates. Small cells are BALB/c anti-EL4 peritoneal exudate CTLs, while the larger ones are EL4 target cells (T) of C57BL/6 origin (Berke et al., ref. 53). A and B: Light microscopy. C and D: Scanning electron microscopy. E and F: Transmission electron microscopy showing contact region and interdigitations. Arrows in D point to interdigitations.



surface multimolecular complex (CD3) (16). These MHC-restricted CTLs can be defined by the rearrangement of their T cell receptor (TcR) α and β genes, the expression of the CD3-Ti molecular complex on the cell surface, and their cytolytic function, which is both Ag specific and MHC restricted. The second type consists of broadly specific CTLs that recognize their targets without MHC restriction, but nonetheless via the CD3-Ti complex. The third type consists of non-MHC-restricted NK cells that recognize and kill certain target cells (NK sensitive) via an as yet undefined receptor. Common to all three forms of lymphocytotoxicity is an initial lymphocyte-target cell adhesion step (conjugation formation) (see Fig. 2), ultimately leading to target lysis. The assessment and mechanism of target cell recognition and lysis is described in a later section of this chapter.

These three distinct effector cell types may also engage in alternative target recognition pathways mediated by exogenously added factors such as antibodies or lectins. For example, in antibody-dependent cellular cytotoxicity (ADCC), the effector lymphocytes express Fc receptors, and target cell recognition is mediated by the interaction of these receptors with the Fc portion of antibody bound to the target cell. Cells capable of performing ADCC have previously been named K cells, but it is now clear that both NK cells and CTLs expressing Fc receptors for IgG (Fc γ R) as well as macrophages and monocytes can mediate ADCC. Non-MHC-restricted, non-specific cytotoxicity can also be induced by CTLs, if the effector and target cells are allowed to react in the presence of antibodies to one or more components of the Ti-CD3 complex or the mitogenic plant lectins concanavalin A (Con A) or phytohemagglutinin (PHA), which provide both effective intercellular bonding and enable triggering of the effector cell lytic machinery.

Cytolytic T Lymphocytes (CTLs)

Effector CTLs are generated in response to allogeneic cell surface MHC determinants (as in allotransplantation), mitogenic lectins, chemically modified or virally infected autologous or syngeneic cells, and tumor-associated Ag. The generation of CTLs from their precursors involves a complex series of events and signals—not all of which are fully understood—ultimately resulting in the production of effectors, capable of specifically recognizing and lysing the target (7,8) (Fig. 3). Schematically, it is believed that resting CD8⁺ CTL precursors (Lyt2 in the mouse) are triggered directly by either foreign class I MHC surface molecules (in allogeneic responses) or by nominal Ag (viral, bacterial) in conjunction with class I MHC molecules (in MHC-restricted responses) (first signal). Within several hours after onset of activation, surface expression of interleukin 2 (IL-2) receptors and blast transformation occur prior to cell division. Proliferation of these activated CTL precursors will not occur unless a second signal, IL-2, is provided. To this end, precursors of T_H cells (CD4⁺; L3T4⁺ in the mouse) are triggered by exposure to allogeneic class II MHC molecules alone (in allogeneic

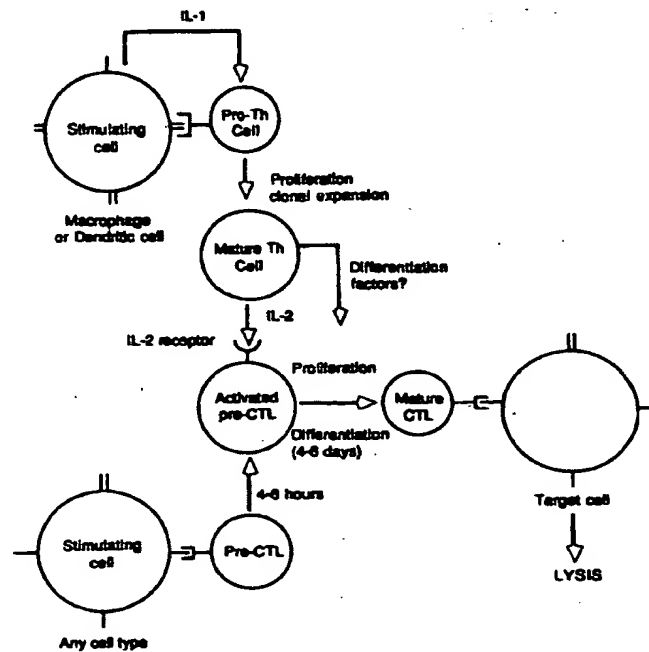


FIG. 3. Cell interactions in the generation of cytotoxic T lymphocytes. Pre-CTLs (lower part of the figure) need at least two signals to become functionally mature. Signal 1 is provided by the interaction of the pre-CTLs' antigen receptor with a MHC class I Ag on an allogeneic stimulating cell (or with foreign Ag presented in conjunction with self-MHC class I Ag in MHC-restricted responses). As a result of this signal, the pre-CTLs become "activated" and express receptors for IL-2. However, the activated CTLs will not divide unless they are provided with a source of IL-2 (second signal). This is normally supplied by a nearby T helper (T_H) cell (upper part of the figure), activated to produce IL-2 by interaction with class II-bearing, IL-1-producing allogeneic stimulating cells, most likely a dendritic cell or macrophage, or with foreign Ag presented in conjunction with MHC class II Ag in MHC-restricted responses. When the activated pre-CTL encounters IL-2, it divides and matures into a fully cytotoxic cell, possibly under the influence of additional (differentiating) factors provided by the T_H cell. The mature CTLs can then attack and destroy cells bearing the same class I antigens as the original stimulating cell. I and II refer to class I and class II MHC antigens, respectively. (Adapted from Wagner et al., ref. 8, and Bach et al., ref. 25.)

responses) or to complexes of class II molecules or nominal antigens (as in virus infection) on dendritic cells or macrophages. Interleukin 1 (IL-1) secreted by the macrophages is presumed to activate the T_H to produce IL-2, which in turn induces proliferation of the activated pre-CTLs (second signal). Under the influence of IL-2 *in vitro*, activated, proliferating lymphoblasts acquire azurophilic granules and serine protease activity. These activated CTLs are cytotoxic and they gradually differentiate into small to medium sized effectors that express potent, spe-

cific cytolytic activity but no cytolytic granules, and finally into memory CTLs. In secondary (anamnestic) CTL responses, the participation of T_h (and IL-2 production) does not appear to be essential for effector (memory) cell activation.

Allospecific CTLs: *In Vivo*

In experimental animals, alloreactive CTLs are generated in response to transplanted allogeneic normal tissues or to transplantable allogeneic tumors (frequently, mouse leukemias are used). One convenient system utilizes the intraperitoneal injection of allogeneic tumor cells where primary rejection of the tumor cells by CTLs can be studied in detail. While spleen or lymph node cells derived prior to, during, or shortly after this allogeneic rejection provide a good CTL source (13), peritoneal exudate cells collected shortly after primary or secondary rejection of an intraperitoneal tumor allograft are an excellent source of highly potent, specific CTLs (17,18). Such cells are capable of binding to and lysing target cells *in vitro*, as determined by the conjugation (target binding) and the ^{51}Cr release (target lysing) assays, respectively (17,18) (Fig. 4).

That MHC class I differences *alone* are required and sufficient for allogeneic CTL generation has been dem-

onstrated by the subtle mutational event(s) of H(Z1) mice—later called bml—where two amino acid substitutions in MHC class I products (class II molecules of the mutant and wild type are identical) give rise to CTL production, cellular alloreactivity, as demonstrated by skin and tumor allograft rejection, and mixed-leukocyte reaction (MLR) (19–21). Interestingly, the cellular alloreactivity and generation of CTLs directed against the mutated MHC class I molecules of bml mutant mice occurs in the absence of detectable alloantibody production (21). Because of distinct cross-reactivities of mutant-specific CTL, it appears that the MHC class I element(s) detected by CTLs in bml mutants is conformational and not sequential (22).

Allospecific CTLs: *In Vitro*

Ginsburg and colleagues were first to generate specific cytotoxic lymphocytes *in vitro* (23). They showed that cocultivation of unprimed lymphocytes with stimulating fibroblast monolayers of various mouse and rat strains (xenogenic and later allogeneic combination) resulted in lymphocyte transformation and production of strain-specific cytotoxic lymphoblasts capable of lysing ^{51}Cr -labeled fibroblast monolayers antigenically identical to the original stimulating monolayers (23,24). Subsequently, it was shown that mixed leukocyte cultures (MLCs) of allogeneic spleen, lymph node, or peripheral blood cells, which have been incubated *in vitro* for a few days, provide an excellent system for studying alloreactivity and CTL production *in vitro* (5,25). Precision was given to this response when it was found that prior treatment of the stimulator cells by mitomycin C or X-irradiation results in one-way MLCs, not unlike MLCs between lymphocytes of homozygous parental and F1 hybrid cells (25). Adoptive transfer of immunity *in vivo* by cytotoxic cells sensitized *in vitro* has been demonstrated (5,26), showing the effectiveness of the *in vitro* primed cells in an *in vivo* setting. CTL generation in MLCs has therefore been used as a well-characterized model system in which both the afferent and efferent phases of T-cell-mediated immunity can be studied. Anamnestic (memory) CTL responses can also be induced and studied *in vitro* by reexposure of resting MLC cells to the original stimulating cell (5), and even to polyclonal stimulators such as mitogenic lectins which result in cell proliferation and prompt reappearance of high levels of specific CTL activity. Moreover, cytotoxic reactivity of these anamnestic CTLs can be induced even when DNA synthesis is completely blocked, for example, by cytosine arabinoside (27).

MHC-Restricted CTLs

Cytotoxic cells specific for a nominal (non-MHC) Ag presented in conjunction with a particular MHC (class I or II) Ag are termed MHC restricted (28–30). Specific MHC-restricted CTLs directed against combinations of class I MHC molecules and small chemical groups [e.g.,

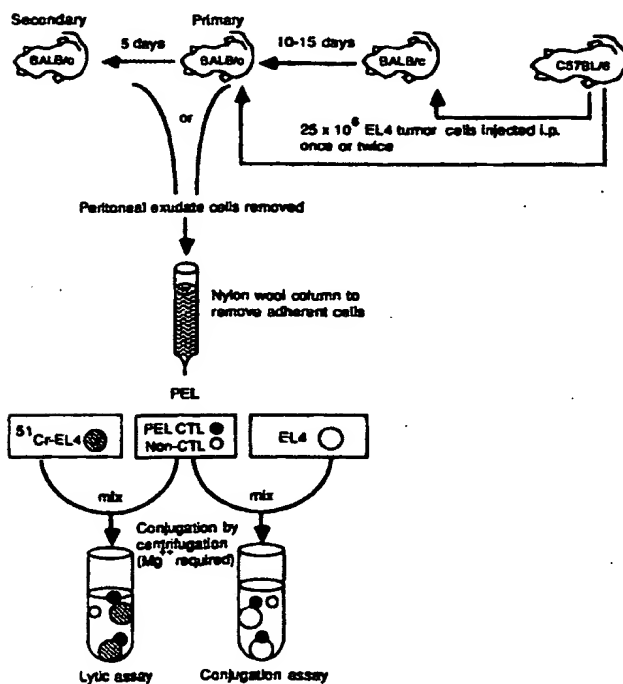


FIG. 4. Generation and testing of the lytic and conjugating activity of *in vivo* primed peritoneal exudate CTLs. (Adapted from Berke, ref. 54, and Berke and Amos, ref. 57.)

dinitrophenyl (DNP), fluorescein isothiocyanate (FITC)] are obtained following *in vitro* cultivation of lymphocytes with autologous lymphocytes modified by the particular chemical (29). MHC-restricted, tumor or virus specific CTLs can be obtained from the lymph node or spleen cells of virus- or tumor-injected animals. The *in vitro* restimulation of these cells with tumor cells or virus-infected cells (28) results in an enhanced CTL response. MHC-restricted CTLs to minor histocompatibility Ags, β_2 -microglobulin, and to other cell surface determinants have also been described (31).

Polyclonal CTLs

Polyclonally activated CTLs can be generated following Con A- or PHA-induced stimulation of lymphocytes. Other polyclonal stimulators include Staphylococcal enterotoxins (e.g., SEB), antibodies to CD3 (a T-cell receptor-associated multimolecular complex), as well as other T cell mitogens. Within 24 hr of such stimulation, small lymphocytes undergo blast transformation and commence cell division. The resultant blasts exhibit a low level of cytotoxic activity against a wide range of target cells (32); this cytotoxicity is enhanced in the presence of the lectin. The low cytotoxicity is because most if not all clones are stimulated; namely, any one clone is represented at a low frequency. Interestingly, *in vitro* stimulation by these mitogenic lectins of memory spleen cells derived from previously alloimmunized mice results in an Ag-like anamnestic response and production of MHC specific CTLs (33,34), probably due to the selective advantage of memory CTLs in response to mitogenic stimulation. Thus lectins can induce both specific and seemingly nonspecific CTLs, the ratios of which depend on previous antigenic stimulation of the responding cells and the system employed.

IL-2-Dependent CTL Lines and CTL Hybridomas

Today, cloned, IL-2-dependent CTLs and NK cell lines (35) are important sources of effector cells for studying (a) the phenotypic expression of differentiation Ag, (b) fine Ag specificity of T cell responses, (c) effector cell activation and cytotoxic activity, and (d) the structure and molecular biology of T cell receptors. One drawback of CTLs and NK clones maintained *in vitro* in IL-2 is that these cultured effectors may develop unexpected specificities (36). For example, cloned CTLs can evolve into cells with NK-like granules and NK-like target specificity (37) but which are not NK cells because they continue to express Ti-CD3 determinants (15).

With the advent of "immortal" antibody-producing B cell hybridomas, the generation of CTL hybridomas became an obvious "next step." Early attempts to somatically hybridize CTLs and tumor cells, thereby forming hybridomas, were unsuccessful. The failure was attributed to polyethylene glycol-mediated CTL lysis of the fusion partner. Nevertheless, successful production of CTL

hybridomas was reported by two groups working simultaneously but independently (38,39). The CTL hybridomas generated by Kaufmann et al. (39) grow without externally supplied IL-2, exhibit specific lytic activity, and express the Thy-1 marker and the T cell receptor (Ti) α and β chains. Although expressing innate lytic activity, the cytotoxic capacity of and IL-2 production by these CTL hybridomas is augmented significantly upon *in vitro* stimulation by mitogenic lectins (e.g., Con A) or specific antigenic cells (40,41), suggesting that the hybridomas are derived from and represent memory CTLs.

Natural Killer (NK) Cells and Lymphokine-Activated Killer (LAK) Cells

These cells, which exhibit LGL morphology, probably play a role in tumor resistance, host immunity to viral and perhaps other microbial infections, and in the regulation of lymphoid and other hemopoietic cell populations (42). NK cells do not exhibit rearrangement of the genes that code for the β chain of the TcR and do not express cell surface CD3 determinants. However, they usually do express CD16 and Leu19 (NKH-1) antigens and Fc receptors. The cell lineage of NK cells is uncertain. The target cell specificity of NK cells suggests that the cytolytic activity of a NK population is not due to a single cell type but is rather the summation of the lytic activities of several different cell lineages at particular stages of maturation and activation. A consistent feature of NK cells which has enabled their isolation, examination, and comparison with other cell types is their association with a subpopulation of cells, the LGLs (reviewed in ref. 42). LGLs are present in the peripheral blood, spleen, and liver of unprimed animals, including athymic "nude" mice and humans. By centrifugation on discontinuous Percoll gradients and elimination of cells that form high-affinity rosettes with sheep red blood cells, a population consisting of 95% LGLs can be obtained from peripheral blood. It should be noted that not all NK cells may be LGLs, nor do all LGLs exhibit NK activity. Certain transplantable tumor cells [e.g., rat NK (RNK) leukemia] exhibit considerable NK activity and cytoplasmic granulation (42,43). Some target cells are highly susceptible to NK-induced lysis (e.g., YAC in the mouse and K562 in humans) while others (e.g., EL4 and Daudi in humans) are refractory. The molecular basis for this differential susceptibility is not well understood. The recent conversion of NK-resistant tumor cells into NK-sensitive targets upon fusion with liposomes containing NK-sensitive membrane determinants (44), or during B cell differentiation (45), may help define the molecular nature of the target cell determinants recognized by NK cells.

Incubation of peripheral blood lymphocytes with IL-2 results in the production of lymphokine-activated killer (LAK) cells, initially thought to be a unique cell population capable of lysing fresh tumor cells but not normal or NK-sensitive target cells (46). Recent evidence suggests that LAK activity can be attributed primarily to IL-2-activated NK cells (47). A separate class of natural cytotoxic (NC) cells, in addition to NK cells, have been

demonstrated in mice. NC cells differ from NK cells with regard to their cell surface characteristics, target selectivity, and organ and strain distribution. In view of the probable involvement of tumor necrosis factor (TNF- α) in lysis induced by NC cells, they may be regarded as effectors of the myelomonocytic (monocytes) series, although highly purified NK cells can also produce TNF (48).

ALLOGENEIC RESPONSES, CLONAL SELECTION, AND MATURATION OF CTLs

The specificity and molecular nature of the CTL T_H receptor are comparable to that of membrane-bound Ig receptors of B cells, although a more complex series of molecular interactions appears to be involved in T cell triggering (16). The application of Burnet's clonal selection and expansion theory to CTLs is complicated by the finding that unprimed animals already contain large numbers of committed T cells reactive to a given alloantigen in mixed lymphocyte reactions. At least one and according to some estimates up to 5% of all T cells from unprimed animals respond to a given alloantigenic challenge. However, a given alloantigenic difference may entail a multitude of antigenic epitopes. Some studies even support the extreme view that no clonal expansion (cell division) is required in an *in vivo* allogeneic T cell response, suggesting that clonal activation alone without expansion may be required and sufficient. However, direct assessment of effector cell replication during CTL production *in vivo* (49) has supported the concept of clonal expansion in the usual type of CTL responses as observed *in vivo*. In that system, the proliferative response of peritoneal exudate CTLs of mice responding to an allogeneic tumor injected intraperitoneally was monitored by administering ³H-thymidine during induction of the CTL response *in vivo*. Almost all the specific conjugate-forming peritoneal CTLs obtained in this case were ³H labeled on autoradiography and thus clearly were the products of dividing cells. Thus the clonal selection and expansion model is applicable to at least one primary *in vivo* alloimmune T cell response resulting in CTL production. However, one must remember that the allogeneic CTL response is biologically artificial; it seems much more likely that MHC-restricted, "self + X" CTL responses *in vivo* would reflect true clonal restriction and expansion à la Burnet.

The observed cytolytic capacity of various lymphoid cell populations, as a function of time after immunization (50–52), is also pertinent to the issue of clonal T cell activation and/or selection. Changes in the lytic potential of a given lymphoid population may be due to alterations in numbers of effector CTLs and/or in their individual cytotoxic activity. A correlation between the number of cells capable of binding target cells and the population's lytic capacity has been demonstrated (Fig. 5). The frequency of peritoneal exudate CTLs capable of binding to and lysing target cells increases from a background of 2–5%, up to 35% within 11 days of primary intraperitoneal alloimmunization and then decreases (53). These findings sup-

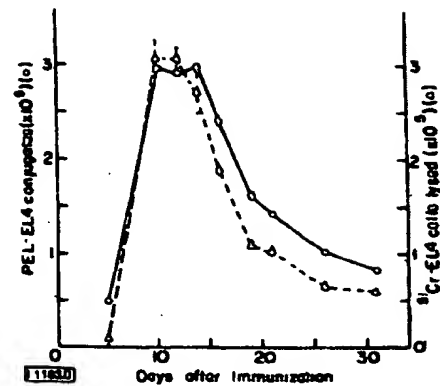


FIG. 5. Conjugation and cytotoxicity as a function of time after a primary alloimmunization. BALB/c mice were inoculated with EL4 intraperitoneally. PELs were isolated at the indicated times and assayed for their conjugation (O) and lytic activity (Δ). (From Berke et al., ref. 53 with permission.)

port the view (54) that alterations in cytotoxic activity following alloimmunization are due to changes in frequency of effector CTLs capable of binding to and lysing the target, rather than to obvious variations in the lytic capacity of individual cells, that is, that effector CTL in an immunized population are either cytotoxic or not (i.e., cytotoxicity is "quantal").

CTL maturation during primary and anamnestic allogeneic responses can be evaluated by examining the specificity of target cell binding by CTLs, the cytolytic and recycling ability of individual CTLs, and the avidity of effector–target cell conjugation (50). The target MHC haplotype and subloci specificity of responding CTLs are preserved after repeated immunizations. Likewise, the lytic rate and recycling ability of individual CTLs are not altered by repeated immunizations. However, inhibition both of CTL–target cell conjugate formation and of CTL-mediated target cell lysis by antibodies against target MHC Ags or the effector's Lyt2 determinants is less effective with tertiary CTLs, suggesting an increase in avidity of effector–target cell interaction after repeated immunization (50). A similar increase in apparent avidity is also observed during CTL priming in MLRs, as deduced from blocking by antibodies directed against CTLs and/or target determinants (27). These observations suggest that responding CTL populations are subjected to moderate selective processes upon repeated antigenic stimuli, and provide a further analogy with B cell responses in which the most avid responders are selected over time and with decreasing amounts of nominal antigen.

IN VIVO FUNCTION OF THE CYTOLYTIC LYMPHOCYTE

Graft Rejection

Early Studies

Multicellular organisms are equipped with an intricate immune system to recognize and neutralize foreign and

potentially hazardous agents, such as intracellular and extracellular parasites and neoplastic cells. Due largely to the work of Snell, Gorer, Medawar, Billingham, and Brent (reviews in refs. 13,55-57), it is now apparent that the rejection of normal (or malignant) allografts is due to a genetically determined and powerful immunological process. That allogeneic tumor fragments enclosed in diffusion chambers implanted into the peritoneal cavities of immune mice were not rejected showed the necessity for direct contact between grafted and host effector cells for rejection to occur (58). On the other hand, antibody and complement can directly lyse certain tumor targets even inside diffusion chambers (59). Mitchison's adoptive transfer experiments (60) demonstrating transference of second set allograft rejections by "primed" lymphoid cells but not by immune allosera established the cellular basis of allograft rejection. Antibody plus complement-induced tissue damage may be a vascular phenomenon, while cellular effectors are involved in response to parenchymal tissue. The "Winn assay" for measuring the antitumor activity of lymphocyte populations by injecting immune lymphocyte-tumor cell mixtures subcutaneously and monitoring tumor growth was an important step in determining the tumor-neutralizing capacity of sensitized lymphocytes in allogeneic and syngeneic ("tumor specific") systems. Govaert (12) was the first to show that canine thoracic duct lymphocytes, procured after kidney allograft rejection, had a specific, cytopathic effect on donor renal cells cultured *in vitro*. His findings were soon followed by a plethora of evidence demonstrating cytopathological effects of lymphoid cells from animals sensitized against normal or malignant tissues, including human cancer, or suffering from autoimmune diseases. *In vitro* lysis of virus-infected cells by cytotoxic lymphocytes obtained from virus-infected animals suggested their involvement in viral immunity and led to the important discovery of MHC restriction.

Allograft-Infiltrating Cytocidal Cells

Involvement of lymphoid cells in the elimination of histoincompatible cells has been established by adoptive transfer and diffusion chamber studies (55,61,62). Infiltration of the graft parenchyma by mononuclear cells, producing a destructive lesion, is a common event both in allograft rejection and in delayed-type hypersensitivity. Depending on the histological type of the transplant, its anatomical site, and the time of examination, the infiltrating cells may be of predominantly lymphoid or macrophage origin.

The cells infiltrating rabbit skin allografts and associated with the damaged graft epithelium are mainly small to medium sized lymphocytes (63). Density distribution analysis of spleen cells from mice undergoing allograft rejection reveals that large, rapidly dividing effector lymphoid cells are present in the spleen shortly after immunization, whereas small effector lymphocytes predominate at later times and during onset of rejection (64). In rodent intraperitoneal ascites allograft systems, where direct investigation of graft-associated cells is relatively

simple, the specific cytotoxic cells at the time of rejection are nonadherent, nonphagocytic, small to medium sized Lyt2^+ T lymphocytes (17,18). The role of the macrophage in this setting varies with different tumor targets from a primary effector cell to a mere scavenger. Interestingly, early (but not later) in the course of an allogeneic response, Lyt2^- negative effectors expressing nonspecific cytotoxic activity have been demonstrated (49). Although they are important topics with respect to tumor destruction, macrophage killing and vascular changes causing necrosis are outside the scope of this chapter.

Small lymphocytes enter the allograft within 1 to 2 days after transplantation and rapidly transform into large lymphoblastoid cells ("peripheral sensitization"); alternatively, sensitization occurs at a draining lymph node. Blast transformation also occurs during mixed lymphocyte reaction, an *in vitro* correlate of allograft rejection (65). That the responding lymphocytes initially undergo transformation into large blastoid cells that express lytic activity, revert to small to medium sized cells that also express lytic activity, and finally evolve into memory CTLs has been demonstrated both *in vivo* and *in vitro* (9,62). Although cytotoxic cells can be detected in lymphatic organs and within grafts shortly after allograft immunization (52,66), graft rejection is usually not apparent until 8 to 10 days after transplantation. The progression of cells into grafts can be studied by the elegant sponge procedure described by Hayry and colleagues (67). When a spongy matrix embedded with allogeneic cells is implanted into a histoincompatible recipient, it is infiltrated by host cells which can be released by squeezing the sponge, thus providing a convenient system for studying allograft infiltration. Although both T and non-T lymphocytes infiltrate the sponge, only a small fraction of the infiltrating cells are T lymphocytes, which is surprising since a potent effector T cell response is observed simultaneously in peripheral lymphoid organs, such as the spleen and lymph nodes. In heart and kidney transplants, several classes of both lymphoid and nonlymphoid cells have been reported to infiltrate the allograft. Unlike the sponge matrix, in these and similar systems, mechanical and enzymatic procedures must be employed to obtain a cell suspension from which the infiltrating cells can be obtained. Naturally these procedures may select for certain cell types, as well as damage or change the properties of the cells in question.

On the other hand, intraperitoneal ascites tumor allografts allow observation of graft-infiltrating cells without any chemical, enzymatic, or mechanical manipulations (68). In such systems, the infiltrating cells, which exhibit specific *in vitro* binding and cytolytic activity at the time of rejection, are small CTLs, although significant numbers of macrophages (up to 50% of the population) and other cell types are associated with the allograft (17). Thus it appears that both large and small lymphoid cells from allografted animals, which exhibit lytic activity *in vitro*, may be responsible for graft rejection *in vivo*.

The T Cell Subset(s) Responsible for Graft Rejection

A large body of evidence indicates that T lymphocytes are the primary mediators of graft rejection, yet the par-

ticular T cell subset involved in the actual rejection process remains controversial (9,67,69,70). The infiltration of CD8/Lyt2 type lymphocytes and the strong cytopathological component in graft rejection have implicated the direct involvement of cytotoxic T lymphocytes (CTLs). Furthermore, a good correlation between several parameters of allograft rejection (e.g., kinetics, genetics, anatomical association) and host CTL activity *in vitro* has been demonstrated. It is possible, however, that the T_H cell subset (CD4/L3T4), involved in delayed-type hypersensitivity (DTH), is at least partially responsible for some tissue damage and graft rejection. The use of antibodies to define functional T cell subsets may be misleading, since the serological division of T cell subsets may not be as restricted as once thought. In addition, some $Lyt2^+/CD8^+$ type cells have been shown to produce IL-2, a T_H characteristic, and some $Lyt2^-$ cells exhibit cytolytic capacities. Furthermore, certain cells can express more than one activity; for example, one cloned T cell line has been shown to express allo-help, cytotoxicity, and DTH capacities (71), and a cytotoxic T cell clone and a monoclonal CTL hybridoma have been shown to generate helper activity (in this case lymphokine production) (40). On the other hand, the observation that cloned $Lyt2^+$ cytotoxic cells can specifically destroy allografts *in vivo* confirms that CTLs can mediate transplantation immunity in an immunologically specific, MHC-restricted fashion (72).

Testing for in Vivo Bystander Cell Lysis During Allograft Rejection

Graft rejection mechanisms mediated solely by a delayed-type hypersensitivity response, involving production of phagocyte-attracting lymphokines or tissue-destructing mediators, which act nonspecifically, rather than by specifically sensitized CTLs, are incompatible with the specificity and selectivity of the rejection episode. For example, skin grafts from allophenic mice (mosaics produced by mixing early-stage allogeneic embryos) grafted onto one parental strain resulted in rejection mainly of the melanoblasts and hair follicles that express the same H-2 as the allogeneic parent type, while those of the host H-2 type did not suffer irreversible damage (73). Furthermore, injection of allogeneic murine tumors mixed with syngeneic tumors (at a cell ratio of 500:1, respectively) resulted in selective and complete rejection of the allogeneic tumor, without affecting the syngeneic one (74). In another CTL system, lymphocyte-induced bystander lysis of cells infected with a third-party virus was also not observed. Thus CTL-mediated lysis is one of the rare cellular cytotoxic mechanisms that displays a high degree of specificity, thereby discriminating between target and bystander cells. However, it is conceivable that, under special circumstances, nonspecific in addition to Ag specific reactions initiated by CTL-target cell interactions may contribute to tissue destruction in allograft rejection, in severe forms of delayed-type hypersensitivity, and in certain viral infections.

Virus and Bacterial Infection: MHC Restriction

Virtually all cells are potential candidates for virus infection and phagocytes are the most susceptible to bacterial infections since they endocytose them. Host cells carrying viruses must be eliminated to keep virus proliferation to a minimum; however, destruction of bacteria- or virus-containing phagocytic cells may result in the dissemination of these organisms. It has been found that immunocompetent host T cells are triggered by bacterial or viral determinants only when they are presented as Ag in the proper molecular form on cell surfaces, in association with MHC class I and class II molecules. Phagocytosed bacteria are digested and their processed Ag are in the form of peptides presented by macrophages in association with MHC class II-encoded surface molecules (MHC class II restriction). These "hybrid" membrane structures induce specific T_H cell proliferation and differentiation, leading to the release of lymphokines, including macrophage-activating factor(s), which ultimately eliminate the infecting bacteria. CTL recognition of viral antigenic determinants on infected cells occurs when they appear in conjunction with MHC class I molecules on the cell surface (MHC class I restriction) (10,28,75). In general class I MHC determinants appear to present endogenously synthesized virus and antigens, while class II is involved in presenting exogenously encountered proteins (76). Anti-influenza CTLs recognize murine L fibroblasts that express viral nucleoprotein (NP) determinants, raising the question of how these originally nontransmembrane viral proteins are processed and presented so that they can be recognized by CTLs. Recent studies are consistent with the view that somatic cells bearing class I molecules are capable of degrading and presenting newly synthesized viral proteins and peptides to CTLs (77,78). The molecular nature of viral epitopes recognized by CTLs on the surface of virus-infected cells is now being resolved (77).

Virus-neutralizing antibodies are effective in preventing initial viral infections. However, cell-mediated immunity is essential to eliminate established viral infections. For example, athymic mice often suffer from persistent and progressive virus infections, probably due to their defective cell-mediated immune system. In murine lymphocytic choriomeningitis (LCM) clearance of the viral infection is effected by CTLs (79). The pathological changes induced by LCM virus have been attributed to damage of virus-infected cells by T cells since virally infected athymic mice do not show brain lesions. In influenza virus infection, whether viral spread is limited by γ -interferon released by CTLs at the site of virus infection, or solely by lysis of infected cells, is not known. However, cloned, influenza virus specific CTLs can protect mice against 10 lethal doses of the specific virus but not against one lethal dose of a non-cross-reacting virus admixed and administered with the virus against which the cloned CTLs were generated (10). These results establish the essential role of direct, specific effector cell contact with infected target cells and exclude involvement of nonspecific cytotoxic factors that work at a distance.

CTLs and NK Cells in Tumor Immunity

Repeated demonstrations of tumor specific immune responses (80–82) have formed the basis of tumor immunology and have supported the theory of "immune surveillance" against tumors. The principal assumption of this theory is that tumor specific immunocompetent host cells can be triggered by neoplastic cells to differentiate into effector cells capable of either destroying the tumor or arresting its growth. Although lymphoid cells exhibiting such effector activity have been observed in experimental animals and in some cancer patients, the precise effector cell populations involved and their specificity have not yet been determined unequivocally and their biological importance is unresolved. Furthermore, cellular immune responses against syngeneic tumors vary according to (a) the host, (b) the tumor, (c) the assays employed, (d) the inoculation site and the dose, and (e) the time and site of effector cell removal.

Analyses of effector cell phenotype, specificity, and mechanism of action against autologous tumor cells reveal that the antitumor cytotoxic response can be multiclonal, mediated by multiple mechanisms, and directed against different determinants expressed on the same tumor cells (83,84). Furthermore, the types and lytic mechanisms of effector cells involved depend on the tumor. For example, using four different syngeneic tumor systems, Haskill and co-workers (80) demonstrated nonspecific, cytostatic macrophages associated with a rat sarcoma; specific, cytotoxic, nonphagocytic, Fc-bearing lymphocytes associated with a murine mammary adenocarcinoma; cytostatic, macrophage-like cells and specific, cytotoxic lymphocytes associated with a primary murine sarcoma, and CTLs within a rapidly growing fibrosarcoma. Adherent, non-T, nonphagocytic cells capable of lysing antibody-coated chicken red blood cells have been detected in the peritoneal cavities of mice with ascites tumors. Nonadherent cytotoxic cells have been obtained from the peritoneal cavities of mice after repeated intraperitoneal injection of irradiated ascites tumors. Even a single intraperitoneal injection of ^{60}Co -irradiated leukemia cells could induce immunity to nonirradiated syngeneic tumor cells in mice (66). In this system, tumor-associated peritoneal exudate cells exhibited specific *in vitro* cytolytic activity as early as 3 days after the intraperitoneal injection of irradiated tumor, and the activity peaked on days 5 to 6.

Tumor Therapy Using LAK Cells and Tumor-Infiltrating Lymphocytes (TILs) Activated by Interleukin 2 (IL-2)

Immunizations against cancers using tumor cell vaccines and attempts to bolster immunity against cancer by immunomodulators have generally been unsuccessful. The commonly low and sometimes even total lack of cytotoxic activity of human lymphocytes against autologous tumors and the difficulty of attaining sufficient quantities

of autologous lymphoid cells whose anti-tumor activity could be boosted *in vivo* or *in vitro* have hindered the development of effective adoptive immunotherapy against human cancer. However, two recent developments have changed the approach to treatment of cancer using immunological intervention. First was the large scale production of recombinant IL-2, previously known as T cell growth factor. Second was the observation that peripheral blood-derived lymphocytes cultured in IL-2 [lymphokine-activated killer (LAK cells)] become cytolytic toward a wide range of fresh neoplastic but not normal cells (46,85). LAK cells, together with high dose IL-2 as reported by Rosenberg and colleagues, have had some success in the treatment of metastatic human malignancies, notably melanoma and renal cell carcinoma (11,82). In some centers, the continuous infusion of cancer patients with lower doses of IL-2 alone without administration of *in vitro* generated LAK cells has yielded comparable therapeutic results.

The LAK phenomenon is mediated by a phenotypically diverse set of effector lymphocytes generated by incubation of peripheral blood leukocytes with IL-2 for 3 days. LAK effectors appear as mostly NK cells, but with time a more T-like phenotype emerges. The majority of LAK cells are derived from NK cells expressing the Leu 19 (NK H-1), but not CD3, surface marker. Peripheral blood CD3⁺ T lymphocytes appear to contribute little to the LAK phenomenon (47). However, under certain circumstances, a particular subset of CD3⁺ NK H1⁺ cells can also be activated by IL-2 and mediate LAK activity.

Recently, Rosenberg (82) reported that lymphocytes infiltrating into tumors could be expanded *in vitro* with IL-2 and used in adoptive immunotherapy. These tumor-infiltrating lymphocytes (TILs) had activity and tumor specificity superior to that of LAK cells. It remains unclear how much of the activity associated with TILs is due to CTL versus LAK and how useful these cells will be in the treatment of human cancers.

Ascites tumors provide an excellent model system to study TILs in experimental animals as well as in humans. TILs procured from the peritoneal cavities of animals that had undergone intraperitoneal allogeneic or syngeneic tumor graft rejection provide a rich source of small to medium sized effector CTLs capable of specific target cell lysis *in vitro* and tumor growth retardation *in vivo* (86). These potent CTLs are devoid of lytic granules, the lytic protein perforin, and BLT-esterase activity. They transform into granule-containing, cytolytic lymphoblasts within several days in culture in the presence of IL-2 (see Fig. 1) (87,88). This observation provides a clue to the cellular and possibly molecular basis for improved efficacy in the immunotherapeutic application of TILs—CTLs expanded in IL-2. Namely, the superb immunotherapeutic activity of IL-2 activated TIL against tumors may be related to their acquisition of cytoplasmic granules in response to IL-2. Proteases and cytotoxic proteins packaged in cytoplasmic granules of IL-2-transformed lymphocytes may be responsible for the antitumor as well as side effects resulting from the administration of large doses of IL-2 *in vivo*.

Tissue Damage in Autoimmunity

Cytocidal lymphocytes seem to play a significant role in inflicting tissue damage in certain autoimmune diseases. This discussion is limited to one autoimmune disease, namely, experimental autoimmune encephalomyelitis (EAE) in rodents. The disease, manifested by nerve conduction defects, can be induced by injecting the animal with myelin basic protein (MBP) or whole brain homogenate in complete Freund's adjuvant. EAE, characterized by massive infiltration of lymphocytes into the central nervous system, is a model system for inflammatory and demyelinating human disorders such as multiple sclerosis. T cells of the helper-inducer phenotype ($CD4^+$) have been implicated as the effectors of EAE, but the mechanism whereby they induce demyelinating damage in the brain, resulting in disease, is not known; furthermore, even the recent production and characterization of encephalitogenic $CD4^+$ T cell lines capable of inducing EAE (89) have not resolved this enigma. Furthermore, the proposed linkage between EAE and delayed-type hypersensitivity induced by $CD4^+$ effectors was recently contradicted by the demonstration that $CD4^+$ encephalitogenic T cells expressing IL-2 receptors can cause EAE in the absence of DTH (90). Recently, cytotoxic $CD4^+$ cells were implicated as causing the onset of symptoms of EAE, possibly by damaging blood vessels in the central nervous system. That Ia-restricted encephalitogenic T lymphocytes lyse autoantigen (MBP)-presenting astrocytes *in vitro* suggests that lymphocytes can directly induce brain damage (91). Studies with the Lewis rat encephalitogenic T cell line (Z1a) capable of inducing EAE in rats have shown substantial lectin (Con A)-dependent cytolytic activity against a wide range of target cells, confirming the proposition that $CD4^+$ Z1a encephalitogenic lines are indeed cytotoxic. Cytotoxic lymphocytes have also been associated with a number of other autoimmune diseases involving lymphocyte infiltrates and tissue damage.

LYMPHOCYTE-TARGET CELL INTERACTION

Binding of Effector and Target Cells

Lymphocyte-induced target cell lysis involves a complex series of events, the first of which is quick specific or nonspecific binding of the CTL or NK to its target, resulting in conjugate formation (Fig. 2). Brondz and co-workers (92,93) originally established the immunological specificity of the physical interaction between CTLs and target cells by demonstrating depletion of cytolytic activity of alloimmune lymphoid cell populations incubated on target cell (macrophage) monolayers genetically similar to the immunizing cells. That this depletion was not due to specific inactivation of the effector cells was demonstrated independently by Golstein et al. (94) and by Berke and Levey (95), who showed that the effector lymphoid cells that adhered specifically to target cell monolayers

could be recovered. The CTLs thus collected were found to have an increased cytotoxic activity against the specific target cell employed for adsorption. Later, Stulting and Berke (96) introduced poly-L-lysine (PLL)-fixed target cell monolayers, to study CTL-target cell binding and showed that Mg^{2+} but not Ca^{2+} was essential for specific CTL-target cell binding. A similar Mg^{2+} requirement was later demonstrated for NK-target cell binding. Since the monolayer adsorption technique examines adhesion of lymphoid populations rather than of individual CTLs, answers to questions concerning the binding of individual CTLs could not be obtained. This has been resolved by the CTL-target conjugation method.

Conjugate Formation

Unequivocal specific binding of CTLs and target cells, resulting in conjugate formation (Fig. 2) was established and termed in 1975 (53,97). It is an early event in the multistep process of lymphocyte-induced lysis. Conjugate formation is induced simply by mixing CTL and target cells in a Mg^{2+} -containing medium (96), spinning to promote interaction, gentle resuspension to break up large cell clumps and weak (non-specific) interactions, and then microscopic examination. Guided by earlier CTL work, NK-target conjugation, and more recently conjugation of T_h cells with Ag-presenting cells, has also been observed. While specific conjugate formation by *in vivo* primed murine CTLs has been repeatedly shown (54), conjugate formation by human and murine CTL lines growing *in vitro* has been found to exhibit considerably less and sometimes even a complete lack of specificity. That conjugated lymphocytes are indeed functional cytotoxic effectors has been demonstrated using single-cell micromanipulation techniques (98). This finding has served as the foundation for a series of experiments aimed at answering questions regarding lymphocyte-target cell interactions at the individual cell level, rather than by extrapolating from population studies.

Specificity, Clonality, and Avidity of Lymphocyte-Target Conjugation

In vivo primed murine CTLs preferentially bind to and form conjugates with target cells displaying MHC determinants identical to, or cross-reacting with, the alloimmunizing cells (53,97). With *in vivo* primed CTLs from the peritoneal cavity of alloimmunized mice (PEL-CTL), the specificity of CTL-target cell conjugation is displayed at all CTL/target cell ratios examined. It appears that up to 40% of immune PEL are capable of specifically conjugating to the immunizing tumor cells. Five to ten percent of these alloimmune peritoneal cells bind nonspecifically to antigenically irrelevant cells (53). Specific binding probably involves the CTL T_i -CD3 receptor complex and

the target MHC class I Ag. The CTL CD8 (or CD4), LFA-1 and CD2 (T11, sheep red blood receptor, LFA-2) membrane determinants also contribute to intercellular binding (99). NK cells preferentially conjugate to NK-sensitive target cells, but neither the NK receptor nor the target determinants recognized by NK cells are known at the present time.

CTL conjugation behaves "clonally" since CTLs from animals immunized simultaneously by two antigenically dissimilar tumor allografts form only little heteroconjugates (i.e., one CTL bound to two different types of target tumor cells) (54). Heteroconjugates, however, can form in the presence of a mitogenic lectin, such as concanavalin A which enables binding of CTLs and third-party target cells. Brondz et al. (93) were the first to demonstrate the generation of distinct subpopulations of alloimmune CTLs against membrane Ag coded for by the MHC H-2D and H-2K subloci by differential immunoabsorption on cell monolayers. A "gene-dosage" effect in the induction of CTLs, as determined by conjugation, is suggested by the results presented in Table 1. Thus alloimmunization of mice with a single peritoneal tumor allograft differing from the host at one or two MHC haplotypes generated 19 and 29% specific conjugate-forming non-adherent peritoneal cells, respectively, while simultaneous intraperitoneal immunization with allogeneic tumors differing from the host at three or four MHC gene loci generated a total of about 48 and 60% conjugate-forming cells, respectively. Lower frequencies of conjugating effectors in a syngeneic intraperitoneal tumor system has been demonstrated (66).

In contrast to specific T cell receptor (Ti)-mediated tar-

get cell binding by *in vivo* primed murine CTLs, antigen specific CTL clones propagated *in vitro* often bind less specifically to target cells although they still kill specifically (99). Three distinct molecular species, namely, LFA1, CD2, and LFA3, appear to be involved in this nonspecific adhesion. The finding that the molecules involved in nonspecific adhesion are also intrinsic to specific CTL-target binding suggests that weak, Ag-nonspecific interactions precede specific interactions mediated by the CTL Ti receptor and the target cell surface Ag (99,100). Theoretical considerations suggest that relatively few receptor-receptor or receptor-ligand bonds may be necessary and sufficient to initiate firm adhesion between cells (101,102). It has been suggested that CD8 and CD4 molecules function to stabilize the interaction between CTL receptors and the corresponding target and/or stimulating cell class I and class II MHC Ags, respectively. Such stabilization may be required by CTLs possessing few and/or low-affinity receptors (103-105) since effector-target cell binding is an equilibrium process (106,107). The avidity of CTL-target cell junctions has been measured directly by determining the force required to separate a conjugated CTL from specific and nonspecific target cells, prior to the delivery of the lethal hit. Interestingly, the force required to break the bonds between specifically conjugated cells (1.5×10^4 dynes/cm²) is about 10 times greater than that required to separate a nonspecific lymphocyte-target cell pair (108). Hence most of the binding force and probably the energy holding a specific CTL and target cell together must come from Ti-CD3 interactions with MHC determinants of the target.

TABLE 1. Gene-dosage effect in CTL-target cell conjugation in alloimmunization

PEL as a CTL source (MHC subloci)	Number of differing MHC subloci	Target cells				Nonspecific conjugation (highest %)	PEL in conjugation ^b per MHC sublocus (estimated)
		EL4 K ^b D ^b	P815 K ^d D ^d	ALB K ^d D ^d	YAC K ^d D ^d		
BALB/c anti-YAC (anti-K ^b)	1	10.5	3.9	4.8	29.5	10.5	19/1
BALB/c anti-EL4 (anti-K ^b D ^b)		42.8	11.8	3.9	8.5	11.8	
C3H/eB anti-EL4 (anti-K ^b D ^b)		46.8	16.0	6.2		16.0	
C57BL/6 anti-YAC (anti-K ^d D ^d)	2	8.2			42.0	8.2	29/2
C57BL/6 anti-P815 (anti-K ^d D ^d)			47.4	35.0			
C57BL/6 anti-ALB (anti-K ^d D ^d)				48.3			
C3H/eB anti-P815 (anti-K ^d D ^d)		10.8	39.5	29.6		10.8	
BALB/c anti(EL4 + YAC) (anti-K ^b D ^b K ^d)	3	40.5			17.5	—	~48/3
C3H/eB anti(P815 + EL4) (anti-K ^d D ^d K ^b D ^b)	4	36.8	34.1			—	~60/4

^a CTL-target cell conjugates were formed by mixing 0.2×10^6 PEL with 10^6 fluorescein diacetate-labeled target tumor cells and scored under a fluorescence microscope.

^b Highest value of nonspecific conjugation was subtracted.

Targeting of Cytolytic Lymphocytes by Lectins and by Antibodies

The exquisite specificity of CTL-mediated lysis, as best demonstrated by MHC restriction of CTL recognition and lysis, can be changed drastically so that CTLs can *non-specifically* lyse virtually any target cell. This can be achieved (a) in the presence of mitogenic plant lectins, such as Con A, or PHA [lectin-dependent cytotoxicity (LDCC)] (32); (b) if the cells are subjected to mild oxidation (109) by periodate (IO_4^-) or by galactose-oxidase [oxidation-dependent cytotoxicity (ODCC)]; (c) by cross-linking the CTL and the target cell with antibodies against the CTL Ti receptor or receptor-associated structures such as CD3 (110) and even through surrogate target antibody inserted in the lymphocyte membrane in a mode that precludes interaction of the inserted antibody and membrane receptors of the effector lymphocyte (111); and (d) by cross-linking Fc-bearing cytotoxic cells to antibody-coated target cells (ADCC) (112).

Lectins are sugar-binding proteins and strong cell agglutinins. It is unclear why only certain plant lectins, notably Con A and PHA, stimulate polyclonal production of cytotoxic lymphocytes *in vitro* and mediate CTL-target cell interactions, resulting in nonspecific lysis. A putative, and unproven, lectin-induced *activation* step (not involving the TcR) following an initial *bridging* (conjugation) step have been proposed as the two signals required and delivered by mitogenic lectins in LDCC (113–115). While mixed cell aggregates of lymphocyte and target cells can be induced by most lectins, only mitogenic lectins appear to induce stable and effective CTL-target conjugation and lysis (Table 2). Furthermore, pretreatment of the target but not of the effector by Con A results in effective cytotoxicity (116). Con A functioning as a "bridge" would be

expected to have a bidirectional role, namely, to induce lysis by pretreated CTLs as well. Therefore it has been proposed that the fundamental mechanism of nonspecific recognition and lysis in LDCC may not be simply through "bridging" and "activation" of the bridged effector by the lectin. Rather it may be analogous to specific CTL-target cell interaction (116,117). That is, the CTL-Ag receptor(s) complex interacts with the target cell surface determinants (including MHC Ag) altered by mitogenic lectins (in LDCC) or oxidants (in ODCC) (116–118). These modifications of target cell surface determinants enable stable, nonspecific conjugate formation ultimately leading to lysis (Table 2). The analogy between specific and lectin or oxidant-dependent T cell lysis is strengthened by the fact that both LDCC and specific CTL-mediated killing are multiphasic, proceeding through a Mg^{2+} -dependent conjugation step, Ca^{2+} -promoted programming for lysis, and a final killer-cell-independent target cell disintegration stage. Furthermore, both specific CTL-mediated killing and antigen nonspecific LDCC or ODCC are similarly influenced by metabolic and cytoskeletal inhibitors, as well as by antibodies directed against CTLs and target cell surface components involved in CTL-target cell interaction (113,115–119).

Antibody-dependent cellular cytotoxicity (ADCC) has traditionally been attributed to "K" cells, macrophages, and NK cells, which bind to antibody-coated target cells (usually red blood cells) through FcR of the effectors. Targeting of cytotoxic lymphocytes (both CTLs and NK) by antibody against either effector or target cell surface determinants is a new, rapidly developing field of considerable theoretical and practical implication. In specific CTL-induced lysis, binding of Ag (or Ag-MHC complexes) on the target cell by the CTL $\alpha\beta$ chains of the heterodimer receptor (TcR) activates the CTL lytic machinery. However, Ti or CD3 specific antibodies attached to various target cells through their Fc portions and the FcR of the target can also trigger the lytic activity of CTLs, leading to lysis of the antibody-derivatized target (110). This reaction probably occurs because of the close physical association of Ti and CD3 molecules in the CTL membrane, as revealed by cocapping and coprecipitation experiments. $(\text{Fab}')_2$ fragments directed against Ti or CD3 do not mediate lysis due to physical proximity, since the Fc portion of IgG (FcR) is involved in the intercellular contact. Ti and CD3 antibody cross-linked to anti-target antibody have also been used to direct CTLs against certain targets. In another system CTL-induced specific lysis of an anti-CD3-producing hybridoma cell has been shown. These last two findings emphasize the role of Ti-CD3 triggering in inducing lymphocyte-mediated cytotoxicity and offer new, exciting means for targeting cytotoxic cells in tumor therapy and virus infection (120–124).

TABLE 2. Lysis and conjugate formation mediated by mitogenic and nonmitogenic lectins

Lectin	Lysis ^a (%)	Number of conjugates ^b
Mitogenic		
Con A (<i>Canavalia ensiformis</i>)	46.0	64
PHA (<i>Phaseolus vulgaris</i>)	77.0	68
LCA (<i>Lens culinaris</i>)	87.0	57
Nonmitogenic		
PNA (<i>Arachis hypogaea</i>)	4.3	1.5
SBA (<i>Glycin max</i>)	14.0	—
PWM (pokeweed mitogen)	6.2	7.5

^a To measure cytolytic activity, polyclonally activated splenocytes of DBA/2 origin were reacted with ^{51}Cr -labeled EL4 as targets. Mitogenic lectins were tested at 2 to 10 $\mu\text{g}/\text{ml}$; nonmitogenic lectins at 20 to 50 $\mu\text{g}/\text{ml}$. CTL/target cell ratio was 10:1. Incubation time was 90 min at 37°C.

^b To measure conjugate formation, peritoneal exudate CTLs were mixed with irrelevant EL4 or P815 cells (1×10^6 cells each), centrifuged at 170g for 10 min at room temperature, resuspended, and the number of conjugates/ $0.5 \mu\text{l}$ was determined.

Quantification of Lymphocyte-Target Cell Interaction Resulting in Conjugate Formation

The term conjugates refers to clusters of effector lymphocytes firmly bound to target cells (Fig. 2) (53). In the

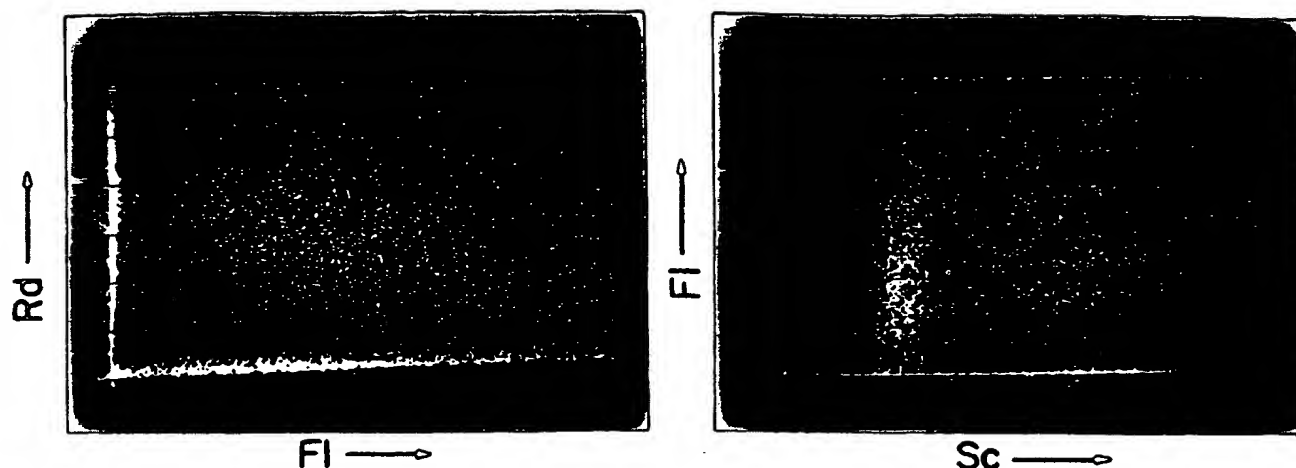


FIG. 6. Analysis of lymphocyte-target cell conjugation by flow cytometry. Fluorescein (Fl) labeled BALB/c anti-EL4 PELs were conjugated with rhodamine (Rd)-labeled target cells (EL4). Left: Rd versus Fl showing conjugates in the diagonal area and nonconjugated effector and target cells on the ordinates. Right: Fl versus scatter (Sc) showing low Sc particles (Fl-PEL); high Sc, low fluorescence particles (EL4); and large, labeled particle (conjugates). (Adapted from Berke, ref. 125.)

conjugation method introduced in 1975 (53,97), suspensions containing CTLs and target cells are cocentrifuged at room temperature to induce conjugation. After thorough yet gentle resuspension, the fraction of lymphocytes bound to target cells is determined in hemacytometers. CTL and target cells can be distinguished by size or by the use of fluorescent dyes. A similar conjugation technique has been applied to NK and T_h cells. To overcome the limitations of microscopic scoring of lymphocyte-target conjugates such as subjectivity, sizes of populations scored, and speed, a cytofluorometric procedure employing a fluorescence-activated cell sorter (FACS) has been developed (125,126). The method is based on the simultaneous monitoring of single-color fluorescence of either prelabeled CTLs or target cells and scatter, or of double-color fluorescence of the conjugated effector and target cells (Fig. 6). The cytofluorometric method has been developed further to sort out specific CTL-target cell conjugates (49), to score conjugate-forming NK cells, and to study single-cell kinetics of lysis.

Competitive inhibition of CTL-target conjugation, assessed by monitoring the impact of unlabeled target cells on conjugate formation between CTLs and fluorescently labeled target cells can be used to evaluate and compare cell surface Ags involved in CTL-target cell interaction (127). Unlabeled homologous target cells lower the frequency of fluorescent target cells in conjugation, proportional to their fraction of the overall target cell population. This method has certain advantages over "cold" target cell inhibition of NK or CTL-mediated lysis, where recycling of effectors influences the linearity of inhibition.

LYMPHOCYTE-MEDIATED CYTOLYSIS

Assessment of Lysis

Target cell lysis following interaction with effector cytotoxic cells can be determined by release of incorporated

radioactive molecules, uptake of dyes which are excluded by intact viable cells, or by end-radiolabeling of the residual cells. Testing the plating efficiency of target cells after interaction with effectors evaluates both cytotoxic and cytostatic effects but is seldom used since it is cumbersome. In 1968, Brunner et al. (128) modified the ^{51}Cr -release assay, long employed to measure survival time of red blood cells as well as complement-induced lysis of nucleated cells, to measure lymphocyte-mediated cytotoxicity. This simple procedure has been used since with virtually no modification since it correlates well with cell lysis and can be used to monitor large numbers of samples. Once incorporated into cells (as $\text{Na}_2^{51}\text{CrO}_4$), spontaneously or actively released ^{51}Cr is not reincorporated, most probably due to changes in the ionic and oxidation state of chromium, binding to aminosugars, peptides, and other cell constituents. For many target cells, the release of the gamma emitter ^{51}Cr from prelabeled target cells provides a good estimate of their viability, correlating quite well with the uptake of trypan blue or eosine dyes. With certain target cells, particularly during extended lytic assays, high spontaneous release of ^{51}Cr complicates interpretation of the results, in which instance alternative radioisotopic procedures (such as the release of the gamma-emitting amino acid ^{75}Se -selenomethionine or of $^{111}\text{Indium}$) or other procedures to determine target lysis must be employed.

With ^{51}Cr (isotopic) release assays, percent lysis is often calculated as follows:

$$(\text{experimental cpm} - \text{spontaneous cpm}) / (\text{total releasable cpm} - \text{spontaneous cpm}) \times 100$$

Although most of the incorporated ^{51}Cr binds to releasable low molecular weight cellular components (3,000 daltons and less), about 25% of all that incorporated binds tightly to high molecular weight cellular components and is nonreleasable even at 100% cell lysis induced by re-

peated freeze-thawing or by exposure to detergents or acids. Depending on the assay conditions, cell type, and assay duration, spontaneous ^{51}Cr release values of 5 to 25% can be obtained. In addition some of the labeled target cells present during lytic assays are not conjugated and occasionally spontaneous ^{51}Cr release due to spontaneous cell death or passive leakage may be substantial (up to 25 to 40%). Hence to compare the lytic activity of populations exhibiting different levels of conjugation, it is important to know the kinetics of lysis solely attributable to effector cells (50). The expression lytic units (LUs) is often used to compare the lytic activity of effector populations (13). One LU is defined as the number of effectors that produce 37% lysis in a unit of time, as deduced from lytic data obtained with serially diluted effectors. Because the extent of lysis is a function of both the concentrations of effectors and of the target, as well as of time, the determination and comparisons of LUs must be done cautiously.

Lysis induced by *individual* effectors conjugated to target cells can be assessed directly by a variety of methods (review in ref. 129). These include single-cell manipulation by micropipettes and phase contrast microscopy (98), examination of the viability (by dye exclusion) of lymphocyte-target cell conjugates incubated in hemacytometers or in agar in the presence of eosine or trypan blue, or recently, flow cytometry of effector-target cell conjugates (125,126,130). Under conditions minimizing effector cell recycling and formation of new CTL-target cell interactions, by using, for instance, highly viscous media (dextran) (131) or by dilution in excess medium, the number of specifically lysed target cells can provide a rough estimate of CTL frequencies.

Dynamics of Lymphocyte-Target Binding, Lysis, and Recycling

Lymphocyte-mediated cytotoxicity is a multistep process initiated by the binding (conjugate formation) of an effector lymphocyte (CTL or NK) to a target cell, delivery of the lethal hit, target cell dissolution, and recycling of the effector (Fig. 7) (57,132). Conjugate formation is a rapid, receptor-mediated process, promoted by cocentrifugation of effector and target cell mixtures (53). The fact that binding is optimal at room temperature, where virtually no lysis occurs, enables the study of this binding stage on its own (132). Dual parameter fluorocytometry has been used to demonstrate that conjugate formation follows first-order kinetics with a half-time of 1.4 min (126). Balk et al. (106,107) have demonstrated that CTL-target cell binding is an equilibrium process. They have also shown the reversal of specific cell-cell adhesion between allogeneic CTLs and ^{51}Cr -labeled target cells. The rate of this reversal appears to depend on the relative affinity of the CTLs to the bound versus free target cells.

Rate of lysis is defined as the number (or density) of target cells lysed by a given number of effectors in a unit of time. Depending on the effector-target system employed, incubation periods of varying lengths are required to detect significant cytotoxicity. For example, CTL can

usually lyse normal or neoplastic lymphoreticular target cells within 1 to 4 hr and even less, whereas fibroblast monolayers may require 12 to 24 hr to lyse. Some CTL populations (e.g., alloimmune peritoneal exudate) kill faster than others and generally the rate of NK-induced lysis is slower than that of CTLs. Additional kinetic considerations are as follows: (a) The rate of lysis induced by either CTLs or NK cells is proportional to the concentration (density) of *both effectors and target cells* (Fig. 8) (57,133). (b) CTLs can *recycle* and kill at the same rate (98,134) at least two to three times (Table 3), whereas functional recycling of NK cells requires reactivation of the effectors (135) although evidence for two cycles of killing by some NK cells has been presented and there may not be a clear distinction between NK cells and CTLs in this regard. (c) Some target cells are lysed as early as 5 to 10 min and others as late as 2 to 3 hr after onset of interaction with the effector (conjugate formation). (d) There is a short *lag phase* (5 to 20 min) before initiation of ^{51}Cr release (13,133). The lag period is not affected by increasing the effector/target cell ratio. However, a delay in ^{51}Cr release from target cells has been observed in both syngeneic systems (136) and when CTL-induced lysis of some target cells (e.g., leukemia EL4 of C57BL/6 mice) occurs in the absence of Ca^{2+} in the medium (137). (e) Temperature has a marked effect on both the rates of conjugation and lysis, although maximum conjugation occurs at room temperatures at which virtually no lysis is detected (53,57). Interestingly, lowering the temperature at an advanced stage of CTL-target interaction (after the delivery of the lethal hit) will completely halt lysis; however, this activity will return to its original rate when the temperature is returned to 37°C (18,138). These results indicate the existence of an intermediate state of a "hit" target which is committed to but not fully lysed.

Escape of CTLs from Self-Annihilation and the Polarity of Lysis

Considerable evidence shows that effector CTLs are not inactivated as a result of deploying their lytic machinery, but that they can recycle to participate in a new lytic interaction (see Table 3) (98,134). Thus any theory on the mechanism of at least CTL-mediated lysis must be compatible with the unidirectionality of the killing process as well as with the recycling of the effector. This does not seem to be the case with human NK cells. Temporary loss of human NK activity as a result of interactions with the NK-sensitive target K562 has been reported although killing activity of the NK population is restored by an IL-2-dependent mechanism (135). If the lytic signal is induced by contact with the effector only and does not involve secretion of a pore-forming toxin(s) into the intercellular gap, then obviously CTL self-destruction would not occur. On the other hand, how effector cells would avoid self-killing, while deploying a "secreted" lytic protein(s), constitutes a dilemma. Hypothetical protective mechanisms have been proposed, but supportive evidence for their existence is not currently available with the exception of homologous restriction factor (HRF) de-

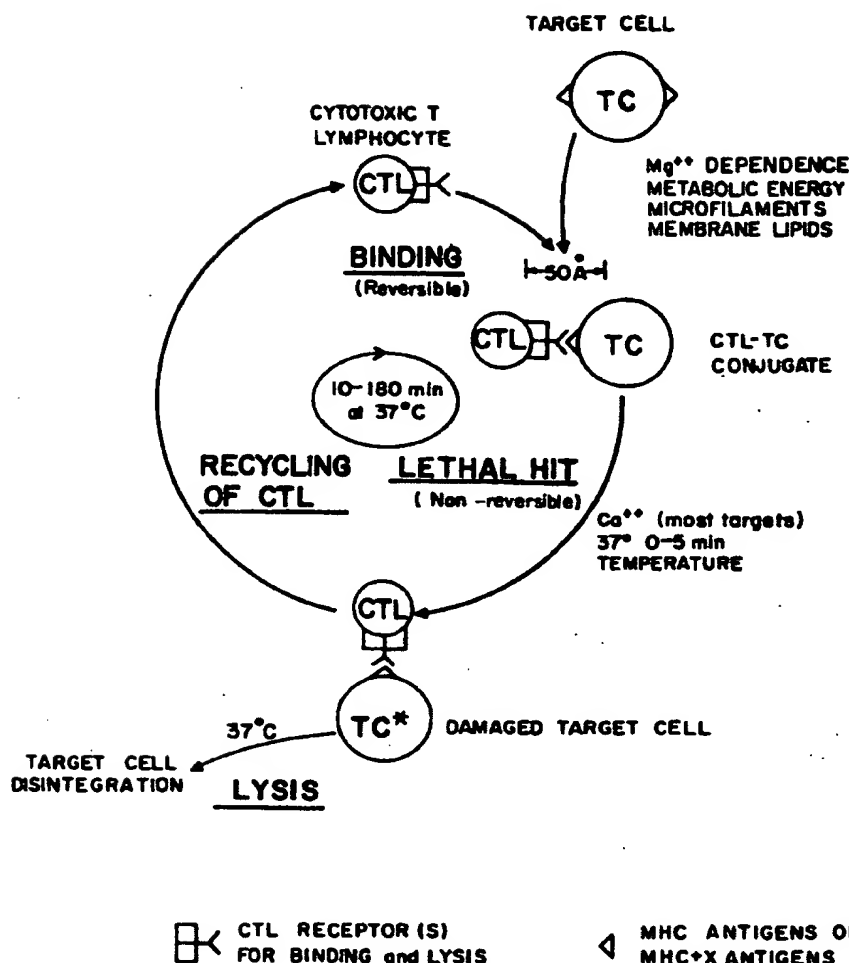


FIG. 7. Lymphocyte-mediated cytotoxicity. (From Berke, ref. 54, with permission.)

scribed by Muller-Eberhard and colleagues. Sparing of the effector CTL is a particularly important issue since, in 1974, Golstein (139) demonstrated that CTLs are not "immune" to attack by showing that B anti-C CTLs can be inactivated (as determined by ability to lyse C target cells) when exposed to A anti-B CTLs (Fig. 9). Interestingly, cloned CTL lines and *in vivo* primed CTLs appear to be more resistant to lysis or inactivation induced by CTLs or by lytic granules as opposed to lysis mediated by antibody plus C (140-143).

That CTLs kill only in the direction of (target) recognition has been deduced from the selective inactivation of only B anti-C CTLs during coinubation with A anti-B CTLs (Fig. 9) (144), as well as from the refractiveness of A anti-B effectors to "reverse" lysis induced by (A \times B) F1 anti-X effectors upon lysis of X target cells (Fig. 9) (145). When studied at the individual conjugate level, unidirectional lysis has been observed even during interaction of mutually immunized CTL populations (A anti-B with B anti-A) (146) although at the population level, as determined by the ^{51}Cr -release assay, bidirectional lysis is observed. Unidirectional lysis at the population level has also been demonstrated in lectin (Con A)-dependent,

nonspecific killer anti-killer lymphocytotoxicity (116). It appears that effector lymphocytes will not express their lytic potential unless their Ag specific receptor complex is occupied by Ag or triggered by Ti-CD3 antibody and will only lyse target cells that are receptor bound (but see ref. 111). Although some conflicting findings exist, bystander cells are usually not lysed, nor are cells bound to killer cell surface Ag other than the Ti receptors or Ti-associated structures such as CD3 (110). However, lysis mediated through CD2 recognition has been reported (the sheep red blood receptor of T cells) (147). The unidirectional lysis of A anti-B or B anti-A CTLs in the course of A anti-B-B anti-A interaction (Fig. 9) may be the result of a head-to-tail type interaction, possibly due to the asymmetric distribution of either CTL surface receptors or cytoplasmic constituents or both. Unidirectional lysis of such mutually immunized CTLs would also occur if the CTL that bound first or more effectively survived the interaction. When a single CTL is bound simultaneously to a number of target cells, the targets are lysed sequentially, not simultaneously (148). This finding also supports the idea of a focal delivery of the lethal hit.

In summary, the lytic event in CTL-mediated lysis ap-

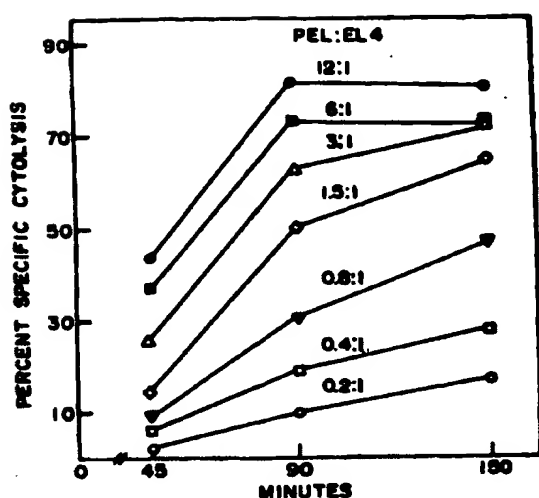


FIG. 8. Time course of lysis of EL4 cells incubated with BALB/c anti-EL4 immune peritoneal exudate cells. PEL:EL4 indicates effector-to-target cell ratios. (From Berke et al., ref. 17, with permission.)

appears to be contingent upon engagement of the CTL Ti-CD3 receptor(s) with a relevant target cell MHC complex Ag and may actually be mediated either by the receptor itself or by associated cell surface components located close by on the membrane or by a secreted component. The receptor-Ag interaction could thus result in target membrane perturbation leading to lysis, or the killer cell receptor could thereby regulate entry into the target of a toxic factor or formation of a membrane channel resulting in cell death. Third-party and bystander experiments have discounted the role of a nonspecific soluble mediator released into the macroenvironment around the killer cell. However, a soluble mediator released upon receptor activation, active only in the intercellular microenvironment and rapidly degraded, thus appearing unidirectional, must be considered.

TABLE 3. Recycling of CTLs: Single-cell analysis by micromanipulation

Experiment	Killers ^a tested	Killers ^b that bound a second time	Killers ^b that lysed a second time
1	10	2	2
2	12	4	3
3	16	3	3
4	10	9	9

^a BALB/c anti-EL4 peritoneal exudate killers were isolated with a micropipette after lysing their conjugated EL4 cells, as determined by trypan blue uptake.

^b Isolated killer cells were placed in contact with intact EL4 cells with the aid of a micropipette and tested for repeated binding and killing activity.

Data adapted from Zagury et al. (98).

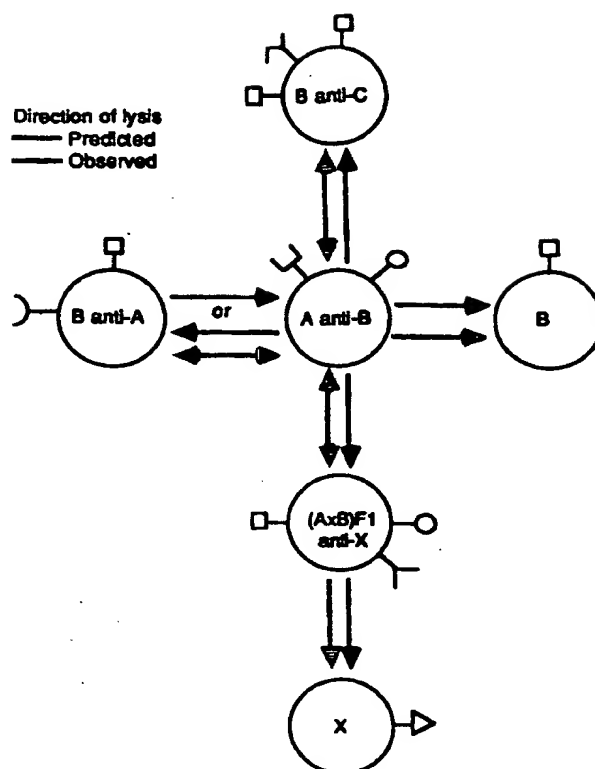


FIG. 9. Unidirectionality of lysis and the role of the CTL receptor in lysis. A anti-B, B anti-A, B anti-C, and (A x B) F₁ anti-X represent specifically immunized CTLs. Y, Y, Y, Y, Y represent receptors for C, B, A, and X antigenic determinants, respectively. □, □, □, represent B, A, and X antigenic determinants, respectively.

Contact Region of Cytotoxic and Target Cells

Electron microscopy of CTL-target cell conjugates has revealed extensive interdigitization of the plasma membranes at the contact region (Fig. 2) (149-151). Formation of fingerlike structures at the intercellular contact zone has also been observed by microcinematography under UV light, of live fluorescein diacetate-labeled CTLs conjugated to live unlabeled target cells. Effector cell projections involved in conjugation contain a network of fine fibrillar material and are devoid of ribosomes, granules, and other obvious cellular organelles. The interdigitization suggests that membrane-folding forces are generated at zones of CTL-target cell contact. This is consistent with the energy dependence of conjugate formation and the requirement of an intact cytoskeletal system (54), unlike the binding of antigen to receptor-bearing B lymphocytes which can occur at 4°C and in the presence of azide. Immunofluorescence microscopy showed that the CTL contact region is enriched with actin but not myosin (152). Anti-tubulin immunofluorescence and EM showed that CTLs usually bind to target cells through a membrane region proximal to the CTLs' microtubule organizing center (MTOC) and centrioles (Fig. 10) (153). A similar



FIG. 10. Localization of the microtubule-organizing center (MTOC) in lymphocyte-target conjugates by tubulin antibody. The MTOC of the CTL (arrow) is proximal to the area of contact, while the MTOC of the target cell is randomly oriented. Bars represent 10 μ m; magnification is 1,200 \times . (Adapted from Geiger et al., ref. 153.)

observation was made later with NK-target cell conjugates (154). It is unclear whether cytoplasmic polarity of the MTOC is related to delivery of the lytic signal proper or to adhesive properties of the cell membrane adjacent to the MTOC, which favor formation of stable intercellular contacts. Proximity of CTL and NK contact regions to the MTOC and centrioles may be related to the general role of the centriole in the effector cell movement toward the target, once contact occurred. For example, it has been shown in motile cells that the centrioles are located in front of the nucleus toward the leading edge of the cell membrane. The membrane in this area of motile cells exhibits an increased protrusive and deformational potential, which may render it more compatible to the formation of stable intercellular contacts. Another CTL organelle found localized in the vicinity of contact regions is the Golgi complex (98,155), suggesting but not confirming the role of a secretory process in conjugate formation and/or target cell lysis or lymphokine secretion. Rearrangement of cytoplasmic granules and other cellular organelles toward the NK-target cell binding site has also been reported (156,157). Based on time-lapse cinematography it has been suggested, but remains to be shown, that after realignment these granules fuse with the membrane and their contents are released into the junctional area of the effector-target conjugate (158) and then are involved in lysis.

Intercellular Communication: Effector Molecules and Secretory Processes in Lymphocytotoxicity

Cell-to-cell communication mediated by intercellular channels is common in organized tissues. Formation of cytoplasmic junctions between effector lymphocytes and target cells, although repeatedly suggested in the past,

was not observed in ultrastructural and tracer studies (149,159). Likewise, freeze-fracture studies did not reveal clear-cut alterations in the distribution pattern of "intramembrane particles" in effector-target contact regions, suggesting but not proving that intercellular membrane fusion does not occur. Thus although unique and temporary communicating membrane substructures may form, failure to detect communicating junctions between the CTL and its target or to demonstrate, by EM, effector cell granules in the process of fusion strengthens the notion that at least certain lymphocyte-induced cytotoxic interactions are strictly contactual and do not involve transfer of material from effector to target through cytoplasmic continuity or granule fusion with the membrane.

The existence of a lytic, or nonlytic, intracellular or secreted material of effector cell origin is not sufficient in itself to establish its role in lymphocytes inducing lysis. However, in recent years, evidence has accumulated suggesting a Ca^{2+} -dependent exocytosis mechanism in at least NK-induced and possibly also CTL-induced lysis. This evidence includes the following:

1. Release of proteoglycans from cytoplasmic granules of human NK cells during target cell lysis (160) and of serine protease(s) during CTL-induced lysis (161).
2. Rearrangement of the cytoplasmic granules, organelles, MTOC, and Golgi of CTLs during lysis (157).
3. Very low NK activity in Chediak-Higashi patients and in beige (bg/bg) mice that both possess mutations causing abnormal lysosomal structure (162). Interestingly, these same beige mice exhibit CTL activity, thus showing the existence of two separate types of lytic mechanisms (NK cells and CTLs).
4. Temporary inactivation of NK cells (but not of CTLs) after lytic contact with target cells (135), suggesting functional secretion by NK but not CTL.
5. Reduction of NK (but not CTL) activity by strontium, known to induce leukocyte degranulation.

6. Inhibition of NK lytic activity by lysosomotropic agents that interfere with lysosomal structure and function, such as chloroquine, NH_4Cl , as well as by drugs (such as Monensin) which affect Golgi-mediated secretion. However, the effects of Monensin on CTL/NK-mediated lysis are complicated by the significant inhibition of CTL-target conjugation (G. Berke and D. Rosen, unpublished results) as well as by the fact that this drug inhibits vesicle fusion to the Golgi apparatus and probably does not influence exocytosis of prepackaged lytic granules as proposed for NK- and CTL-induced lysis. Furthermore, lysis in the absence of Ca^{2+} in the medium, a condition which prohibits granule exocytosis, as well as perforin-induced cytotoxicity has been demonstrated (137,163).

Subcellular and Soluble Cytolytic Factors in Cell-Mediated Cytotoxicity

There are indications for a secretory process in lymphocytotoxicity and several lytic factors have been obtained from the culture medium of resting or activated cytotoxic lymphocytes or extracted directly from the cells. Cytolytic lymphocytes can produce two different types of cytotoxic effector molecules, namely, granule-associated proteins that can induce prompt, nonspecific cell lysis and other non-granule-associated secreted proteins that can cause protracted (12 to 24 hr) but selective lysis.

NK Cytotoxic Factor(s) (NKCF)

This slow-acting factor(s) is released from murine, rat, or human NK cells during their interaction with NK susceptible targets. Release of NKCF can also be induced by mitogens. Because the factor(s) retains both the species and target specificity of NK cells, it is believed to be involved in target cell lysis mediated by NK cells (Fig. 11) (164). NKCF is absorbed by susceptible target cells through putative NKCF binding receptors, possibly cell surface glycoproteins/glycolipids. Its internalization appears to induce irreversible damage to the target by an unknown mechanism, resulting in target lysis without further exposure to NK cells. Clearly more work is required to establish the precise molecular nature and function of NKCF in NK-induced lysis.

Lymphotoxin (LT) (TNF- β)

Activation of B or T lymphocytes with Ags or mitogenic lectins can lead to production of LT (also called TNF- β), a tumor necrosis factor (TNF- α)-like molecule of 171 amino acids (~18 kd) which can nonspecifically lyse certain sensitive target cells (165-167). However, *in vivo* (74) and *in vitro* (168) experiments with bystander cells to detect diffusible effector molecules have indicated that LT may not be involved in CTL-mediated lysis. Furthermore,

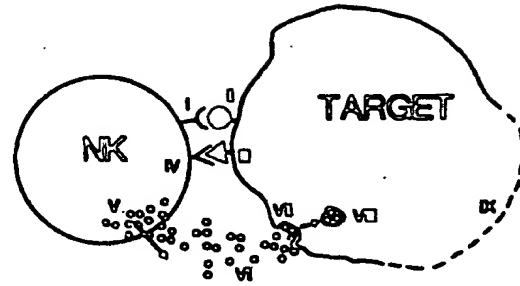


FIG. 11. Model proposed for the mechanism of lysis induced by NK cells. (I) NK recognition structure. (II) NK target structure(s). (III) Stimulating target cell structure. (IV) NK receptor. (V) Release of NKCF. (VI) Released NKCF. (VII) NKCF binding site. (VIII) NKCF processing. (IX) Target cell death. (Adapted from Wright and Bonavida, ref. 164)

LT antibodies which retard LT activity in a delayed-type hypersensitivity reaction do not affect CTL-induced lysis (168). However, the possibility has not been excluded that LT or another lytic product is secreted into the intercellular space or transferred from CTL to target cells through specialized junctions, following specific CTL-target cell conjugation.

Tumor Necrosis Factor (TNF- α)

This toxic factor is found in the serum of animals infected with *Bacillus Calmette-Guerin* (BCG) and endotoxin (167,169,170). TNF- α is capable of inducing hemorrhagic necrosis of murine tumors, particularly when injected into the tumor lesion. It is a nonglycosylated protein of 157 amino acid residues (~17 kd). TNF- α is produced by macrophages, and macrophage-induced lysis is blocked by TNF- α antibodies. Neither TNF- α nor TNF- β antibodies blocks lysis induced by NK cells. TNF- α is structurally homologous to LT (TNF- β) and exhibits 30% amino acid homology. TNF- α and LT interact with cells through a specific class of high-affinity receptors ($K_d < 10^{-10}$ M), the number of which is increased by interferon. Both TNF- α and TNF- β (LT) are cytotoxic to only a narrow range of cells, with L929 cells being the most susceptible target.

Lytic Granules, Cytolysin/Perforin, and Serine Proteases

NK cells and some but not all CTLs, particularly those cultured with IL-2 or responding to stimulation by Ags/mitogens or IL-2, contain defined cytoplasmic granules, which have been observed by EM (Fig. 12), isolated, purified on Percoll gradient, and analyzed (43,171-173). These cytoplasmic granules contain the Ca^{2+} -dependent lytic protein(s) perforin/cytolysin, hydrolytic lysosomal enzymes, and several distinct serine proteases and proteoglycans (~200 kd). Ca^{2+} -dependent exocytosis of

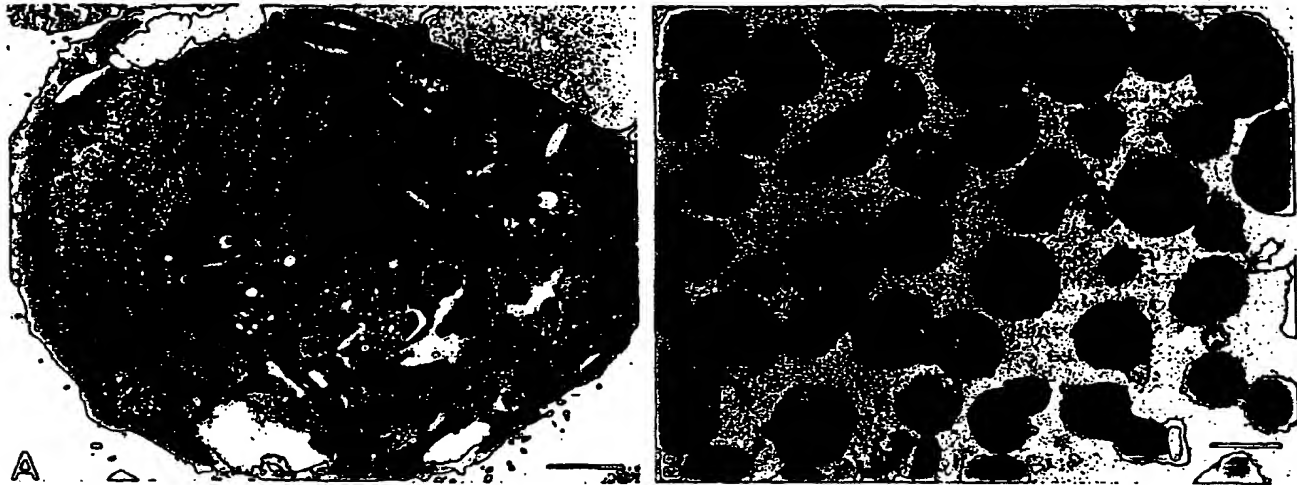


FIG. 12. Electron micrographs of a granular CTL and its isolated granules. A: A CTL-2 cell. Note abundance of osmiophilic granules (gr). Bar represents 1.2 μ m. mit, mitochondria. B: Purified granules extracted from CTL-2 cells. Bar represents 526 nm. Electron microscopy by D. Rosen.

granule contents has been proposed to be involved in target cell lysis induced by cytotoxic lymphocytes. At least one and possibly two proteins of 70 to 75 kd (reduced) are present in purified granules extracted from both NK cells and some CTLs that contain such granules. They are antigenically related to the ninth component of complement (C9) (171,174), but unlike C9, cytolytic/perforin in itself causes rapid and Ca^{2+} -dependent lysis of a variety of target cells, including red blood cells as well as nucleated cells which are NK resistant (175). Both human and mouse perforin have been recently cloned, the genes exhibiting 30% homology to C9. Cytolytic/perforin induce the disintegration of liposomes as determined by rapid release of internalized carboxyfluorescein (43). When polymerized, perforin forms characteristic hollow tubular structures (I.D. 150 to 200 \AA) spanning the membranes of the target cells (171). Negatively stained and examined by EM, these structures appear as "rings" embedded in the membrane (Figs. 13A and 14). Functional transfer of perforin from granulated effector CTL to target cell has been inferred in one report from the immunofluorescence of perforin antibody applied to CTL-target cell conjugates (171). Curiously, the antibodies directed against perforin, which effectively block lysis induced by purified perforin or perforin-containing lytic granules, do not block lysis induced by *in vivo* primed CTLs, hence direct proof for the involvement of perforin/cytolytic in CTL-induced lysis is lacking.

A few distinct CTL specific serine esterases have been discovered both at the gene and at the protein levels (176-179). These include CTLA1 (also termed CCP1 or granzyme B) and CTLA3 (H factor, granzyme A). Lytic cytoplasmic granules can be extracted from CTL-NK cell lines and appear to be the intracellularly localized storage organelle of these enzymes. The enzymes are released upon specific CTL-target cell interaction, resulting in

lysis (179,180), but their role, if any, in inducing lysis is as yet unknown (181). Recently we observed transient expression of serine-proteases during primary but not secondary CTL response *in vivo* (Gardyn and Berke, *unpublished*), suggesting that CTL transiently acquire granules containing serine-proteases in the course of their primary stimulation, probably under the influence of IL-2. Hence protease activity of CTL correlates with their stage of differentiation rather than cytotoxic activity. Two newer CTL genes, CTL-A2 α and CTL-A2 β , appear to have the pro-region of a cysteine protease whose function in inducing killing is also unknown at the present time.

THE "LETHAL HIT" AND THE MECHANISM OF LYSIS

Early Studies

Several theories have been proposed for the nature of the lethal hit and the mechanism of lymphocyte-induced lysis (reviews in refs. 2-4,182-184). The apparent sequential release of cellular constituents from the affected target according to molecular size, combined with the protective effects of high molecular weight dextrans (185,186), supported the theory of small lymphocyte-induced target membrane damage, ultimately leading to colloidal-osmotic swelling followed by target cell membrane rupture (187). Alternatively, the firm adhesion of CTLs to target cells was proposed to cause tangential shear force on the target cell membrane, leading to target cell damage and lysis (188,189). Yet another theory proposed the activation of a membrane-bound phospholipase resulting in removal of one fatty acid from phosphatidylcholine, thereby causing formation of lysophosphatidyl-



FIG. 13. Membrane-bound "ring" structures in immune cytotoxicity. A: Membranes following lysis induced by hyperimmune serum + complement (BALB/c anti-EL4 serum plus EL4 target cells). Typical rings are seen (arrow). Bar represents 43.8 nm (A), 55.5 nm (B), and 47.6 nm (C). B: Membranes following NK-induced lysis (human peripheral blood LGL plus K562). Typical rings are seen (arrow). C: Negatively stained membrane isolated after CTL-induced lysis (BALB/c anti-EL4 PEL plus EL4 target cells). No rings are seen. Electron microscopy by D. Rosen.

choline (lyssolecithin), a strong detergent and a potent cytolytic agent, capable of destroying the integrity of the target cell membrane. Phospholipases generally do not attack the phospholipids of intact plasma membranes; however, they may cleave membrane phospholipids in the presence of membrane-perturbing factors, such as detergents or a basic snake venom peptide and possibly during CTL-target cell interaction. As plasma membrane fractions from stimulated lymphocytes were found to lyse target cells, it was suggested that physical contact with the effector cell membrane in itself may be sufficient to induce target lysis. However, linkage between CTL recognition and lytic activities, as manifested by lack of lysis of A anti-B upon interaction with B anti-C CTLs (144), suggests that the mere close apposition of CTL and target cell membranes, although required, is insufficient to cause lysis (Fig. 9).

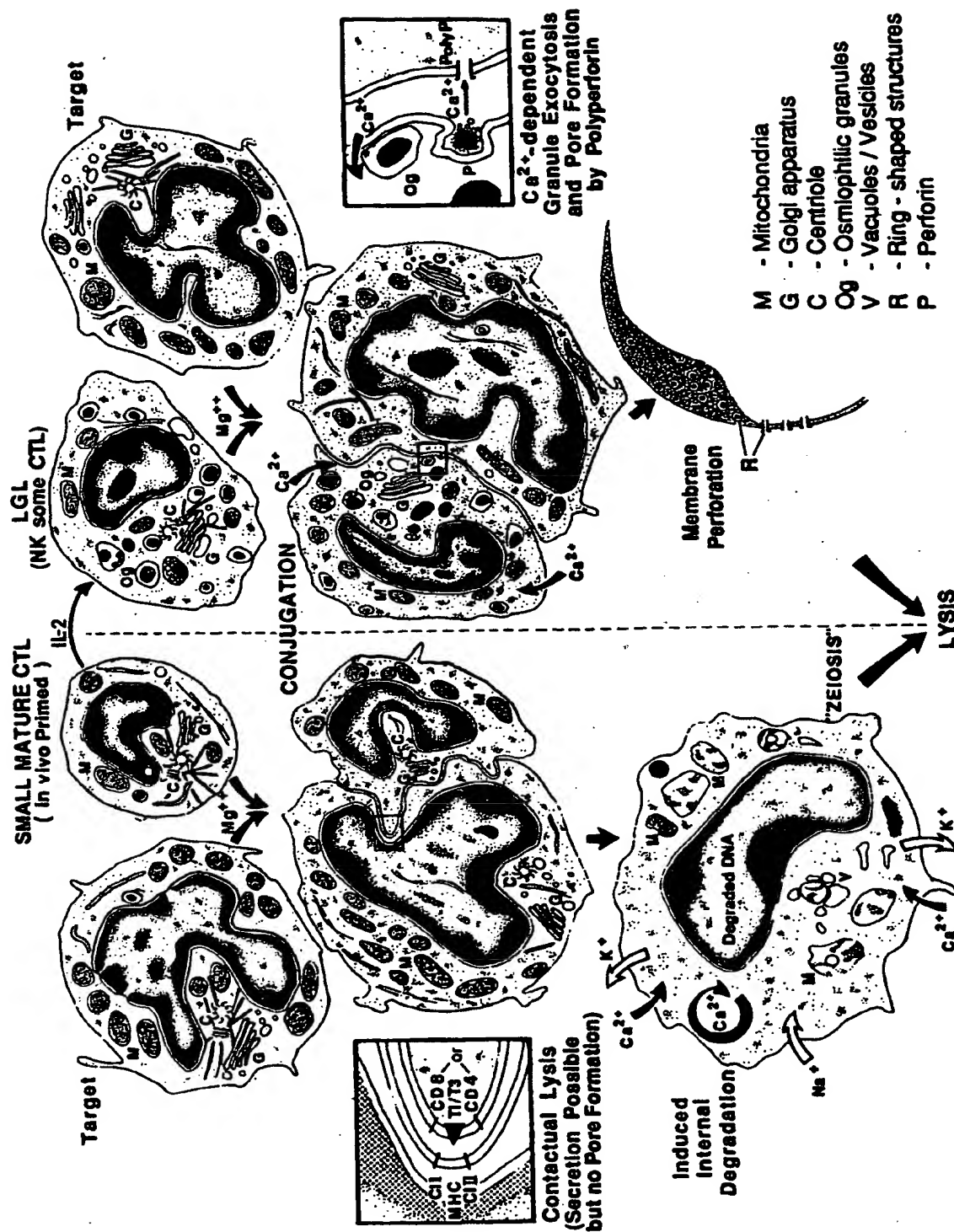
Current Studies and Theories

Due to some common characteristics and the same end result (cytolysis), there is a recent tendency to propose a common lymphocytotoxic mechanism, regardless of the effector cell-type involved (43,171,190). Such an example is the proposed mechanism of lymphocytotoxicity mediated by exocytosis of lytic granules and formation of 10- to 20-nm pores in the target cell membrane. However, the elucidation of certain defined, prelytic events and the careful analyses of *in vivo* primed effector cell populations devoid of lytic granules and perforin suggests the existence of more than one lytic pathway in lymphocytotoxicity (191-194). Furthermore, target lysis induced even by granule-containing lymphocytes (CTLs or NK cells)

can occur in the absence of granule secretion (163,195). Interestingly, lytic granules can be induced in highly potent *in vivo* primed peritoneal exudate CTLs devoid of such granules, upon cultivation *in vitro* in IL-2 (87,88). Taken together, the recent results suggest that either granule exocytosis is not involved in inducing lysis, or that the effector cells can lyse targets by two different lytic mechanisms. Currently, two nonmutually exclusive theories exist to explain the mechanism whereby various CTLs and NK cells induce lysis: (a) that a pore-forming protein(s), packaged in cytoplasmic granules and secreted upon CTL or NK interaction, induces lysis of the target (43,171,174) and/or (b) that a transmembrane stimulatory signal(s) delivered to the target upon receptor-mediated effector-target interaction induces internal disintegration of the target (191-194). The two theories, which probably apply to different effector cell types, are schematically illustrated in Fig. 14 and are discussed next.

Granule Exocytosis and Formation of 100- to 200-Å Pores in the Target Membrane

This theory (43,171) stems from the observation that NK cells and certain CTLs possess lytic cytoplasmic granules and the discovery of complement-like lesions on membranes of lysed cells. It has been suggested that target cell binding to a specific NK cell and to certain CTL-membrane receptors induces a secretory process in the effector cell which results in the contents of the lytic cytoplasmic granules being released. It is these lytic components in the localized environment which have been proposed to cause target cell lysis. NK- and CTL-mediated lysis would thus be the end result of perforation of target cell membranes. Indeed negative staining electron microscopy of lysed targets in some experiments has



shown protein-lined transmembrane holes (internal diameter, I.D., 100 to 200 Å) which appear as membrane-bound "rings" (Figs. 13A,B and 14). They are believed to be structurally and functionally analogous to target cell lesions produced by the membrane attack complex of complement (Fig. 13A) (171,196). Cytoplasmic granules of CTLs and NK cells, which contain the lytic protein(s) perforin/cytolysin (~70 kD), lysosomal enzymes, and serine protease(s) activity, have been proposed to be the origin of the ring-forming material(s). Ca^{2+} has been claimed to be necessary for (a) the induction of exocytosis of the lytic granules and (b) inducing polymerization of the lytic protein perforin (perforin $\xrightarrow{\text{Ca}^{2+}}$ polyperforin), believed to be involved in the perforation of the target membrane (see Fig. 14, right-hand box). However, the demonstration of CTL-induced cytotoxicity in the absence of Ca^{2+} (137) would seem to invalidate this mechanism and suggest an alternative pathway.

Effector Cell Triggering of Target Disintegration Not Initiated by Pore Formation

Several observations are not consistent with a single mechanism of CTL- and NK-induced lysis, mediated solely by perforation of the target cell membrane with 100- to 200-Å "holes" by effector granule constituents:

1. Perforin/cytolysin has been detected in nonlytic cells, while certain *in vivo* primed, highly potent CTLs derived from the peritoneal cavity of alloimmunized mice lack perforin or lytic granules or both, and they do not induce complement-like "rings" in the lysed target membrane (Fig. 13C)

(43,88,191,197-199). Also, most cloned CTLs do not seem to have detectable perforin (199).

2. CTL-induced lysis of some target cells can occur in the absence of Ca^{2+} in the medium (137), a condition in which no exocytosis of granule constituents is demonstrable (163,195), and perforin/cytolysin is nonlytic to cells since perforin-induced toxicity is strictly Ca^{2+} -dependent (43).
3. The prelytic increase in cytosolic Ca^{2+} in target cells (137,191) and the disintegration of the target DNA into 190-bp units prior to ^{51}Cr release (194,200,201) are difficult to reconcile with a lytic mechanism initiated by the formation of "holes" of I.D. 100 to 200 Å in the target membrane!
4. The release of ^{51}Cr , nicotinamide, amino isobutyric acid, and even ^{86}Rb from CTL-damaged target cells is temperature dependent and is fully arrested at 7°C and even 20°C (18,138), while leakage from damaged targets through putative protein-lined "holes" (I.D. 10 to 20 nm) should be only minimally affected by temperature, if at all.
5. Antibodies against lytic granules and/or components thereof (such as perforin/cytolysin) block lysis induced by cytolytic granules but not by *in vivo* primed peritoneal exudate CTLs and other CTLs (202).

The alternative

In view of these intriguing findings, an alternative lytic mechanism must exist. Lymphocyte-induced internal disintegration of the target cell (autolysis) has been suggested by some investigators (192,194). We shall now discuss the possible nature of such an inductive signal delivered by

FIG. 14. Schematic illustration of two currently proposed pathways in lymphocyte-mediated lysis. Contactual lysis inducing self-disintegration (left) and granule exocytosis resulting in pore formation (right). Killing by contactual lysis (left side of scheme) is effected by *in vivo* primed, small to medium sized (7 to 10 μm) CTLs devoid of lytic granules and perforin and which exhibit only background levels of BLT-esterase activity. These effectors are MHC-restricted, T1/T3-positive CTLs. In the presence of Mg^{2+} , these CTLs bind target cells, thus forming conjugates. The CTL, MTOC and Golgi complex are oriented toward the contact area, which is characterized by membrane interdigitation. Under permissive ion and temperature conditions, a cascade of events is initiated in the target, culminating in zellolysis and cytotoxicity. These include target membrane derangement caused by CTL binding, which results in K^{+} efflux (depolarization), and an increase of cytosolic Ca^{2+} (from external/internal stores). Ca^{2+} may be involved in inducing prelytic DNA degradation, damage to the nucleus and mitochondria, activation of ATPases, and ATP depletion, blebbing, and membrane damage culminating in zellolysis. The effector cell can recycle without reactivation. In the lytic granule exocytosis pathway (right side of scheme), the killer is a large (10 to 20 μm) granular lymphocyte (certain CTLs and NK cells). Some of these effectors are T1/T3+, MHC-restricted, while others (including NK cells) lyse nonspecifically. These effector cells are characterized by lytic cytoplasmic granules containing perforin and BLT-esterase(s). When presented with an appropriate target, the granular effector cell undergoes a series of events, similar to those with agranular killers, to form interdigitated contact with the target. This is followed by external Ca^{2+} -dependent granule exocytosis and release of a pore-forming protein(s) (perforin/cytolysin) into the intercellular gap. The released perforin undergoes Ca^{2+} -dependent polymerization to form a protein-lined hole (I.D. 100 to 200 Å) perforating the target cell membrane. These lesions appear as ring-shaped structures in negative-staining EM. Other granule and nongranule components (NK cytolytic factor (NKCF), $\text{TNF-}\alpha$, $\text{TNF-}\beta$) may contribute to target lysis. This effector cell type requires reactivation before it can interact with and lyse another target cell.

the killer and how target lysis is achieved (scheme, Fig. 14, left-hand side). It has been proposed that CTL-mediated lysis is initiated by multiple submicroscopic molecular derangements in the target membrane structure (191) upon lymphocyte-target interaction. Membrane derangements may result from direct contact of the target MHC and/or other cell surface determinants with the CTL Ti-CD3 complex (Fig. 14, left-hand box) and involve other membrane-bound (e.g., CD8 or CD4) or secreted lymphocyte effector molecule(s) (192). Under permissive temperature and ionic conditions, a multitude of such intercellular contacts would induce membrane depolarization as measured by $^{86}\text{Rb}^+$ (a K^+ analog) efflux from the target, simultaneously with the delivery of the lethal hit (131,203). Subsequent permeability changes observed in the target following contact with CTLs probably reflect progressively failing ionic pumps, which are only initially capable of controlling K^+ efflux (depolarization) and Na^+ and Ca^{2+} influx through the deranged (depolarized) membrane.

The outcome of any low-level chemical, physical, or immunological (complement- or CTL-induced) damage to targets depends on that cell's ability to compensate for and/or repair the damage inflicted. Small perturbations in ion fluxes are dealt with by enhanced outward ion, mainly Na^+ and Ca^{2+} , pumping activity, energy metabolism, and oxygen consumption by the affected cell. Substantial, or small but persistent, derangements of the cell membrane can exhaust energy resources involved in ion pumping. Cessation of outward Na^+ pumping ultimately leads to net water influx, colloidal-osmotic cell rupture, and target disintegration. On the other hand, the prelytic, postlethal hit loss of cellular K^+ occurs with the concurrent entry and accumulation of Na^+ . Although the transmembrane ion concentration gradients are thus dissipated, the intracellular concentration of colloids (proteins) is still higher than that present externally, and the cells thus begin to swell slowly. Hence disruption of the Donnan equilibrium may facilitate net uptake of water, swelling, lysis, and release of high molecular weight compounds (131,203,204).

The role of cytosolic Ca^{2+} in inducing cellular injury

The above sequence of events does not account for certain prelytic effects (preceding ^{51}Cr release) characteristic of lymphocyte-induced cytotoxicity. These include DNA disintegration, cytoplasmic streaming, blebbing, and finally "zeiosis" (cell boiling). The prelytic elevation of cytosolic (and probably nuclear) Ca^{2+} levels (which can be detected within minutes of lymphocyte-target cell interaction) has been suggested to be an essential prelytic event, inducing internal disintegration processes in the target cell which culminate in zeiosis (137,191,192). Increase in Ca^{2+} may contribute to DNA fragmentation (194,200), through triggering of Ca^{2+} -dependent topoisomerases and nucleases, which induce uncoiling and fragmentation of the DNA, respectively. Target cell protease activity induced or enhanced by Ca^{2+} could damage cy-

toskeletal elements resulting in bleb formation (205) and finally zeiosis. Mitochondria were shown to be one of the first cellular organelles affected in the course of lymphocyte-mediated cytotoxicity. Energy production by mitochondria could be suppressed by Ca^{2+} -induced damage to mitochondrial structure/function, affecting generation of ATP and thus the function of ATP-fueled ion pumps. Ca^{2+} enhancement of phospholipase activity may induce damage to the plasma and internal membranes. Ca^{2+} activation of major cellular ATPases, such as actomyosin, could lead to massive ATP and phosphocreatine depletion, further suppressing Na^+/K^+ -ATPase (sodium pump) activity, already compromised by the depolarized membrane and decreased mitochondrial ATP production (191,192).

IL-2-Induced Acquisition of Cytocidal Granules, BLT-Esterase and Perforin mRNA by Lymphocytes and the Two Pathways of Lymphocytotoxicity

The demonstration of complement-like "holes" (I.D. 100 to 200 Å) on membranes of targets lysed by effector lymphocytes and of lytic granules and perforins, in certain cytotoxic lymphocytes (immature CTL *in vivo*, LGL or CTL cultured in IL-2), led to the suggested mechanism of lytic granule exocytosis and a common terminal step in lymphocyte and complement-induced lysis. However, neither the formation of complement-like "rings" in targets were caused by, nor have lytic granules, perforin/cytolysin, or serine esterases been found in either highly potent, mature peritoneal exudate CTLs (day 11 after primary or day 5 after secondary immunization), in cytotoxic hybridomas generated from them (PEL hybridomas), or in a number of other CTL lines (88,197,198). We have recently found that upon incubation *in vitro* in the presence of IL-2, the small *in vivo* primed cytolytic PELs transform into large, dividing cytolytic T cells (PEL-blasts), which express authentic PEL specificity in short-term lytic assays (87,88). PEL-blasts, in contrast to the small *in vivo* primed PELs, possess massive quantities of lytic granules (Fig. 1), serine esterase activity (Table 4)

TABLE 4. Expression of protease (BLT-esterase) activity in various CTL

Cells	BLT-esterase activity ^a
CTL-2 (IL-2 dependent, <i>in vitro</i>)	6.00
PEL-CTL (4 days after <i>in vivo</i> priming) ^a	0.540
PEL-CTL (11 days after <i>in vivo</i> priming)	0.228
PEL-CTL (5 days after <i>in vivo</i> priming)	0.019
PEL-CTL hybridoma	0.056
PEL-Blasts (IL-2 dependent, <i>in vitro</i>)	6.8–47.0 ^b
Normal spleen	0.222
EL4 leukemia	0.220
P815 leukemia	0.386
L1210 leukemia	0.018

^a OD 412 nm/1 × 10⁶ cells/15 min.

^b Various cultures.

^c Transient expression of BLT-esterase during primary but not secondary PEL-response (Gardyn and Berke, *in preparation*).

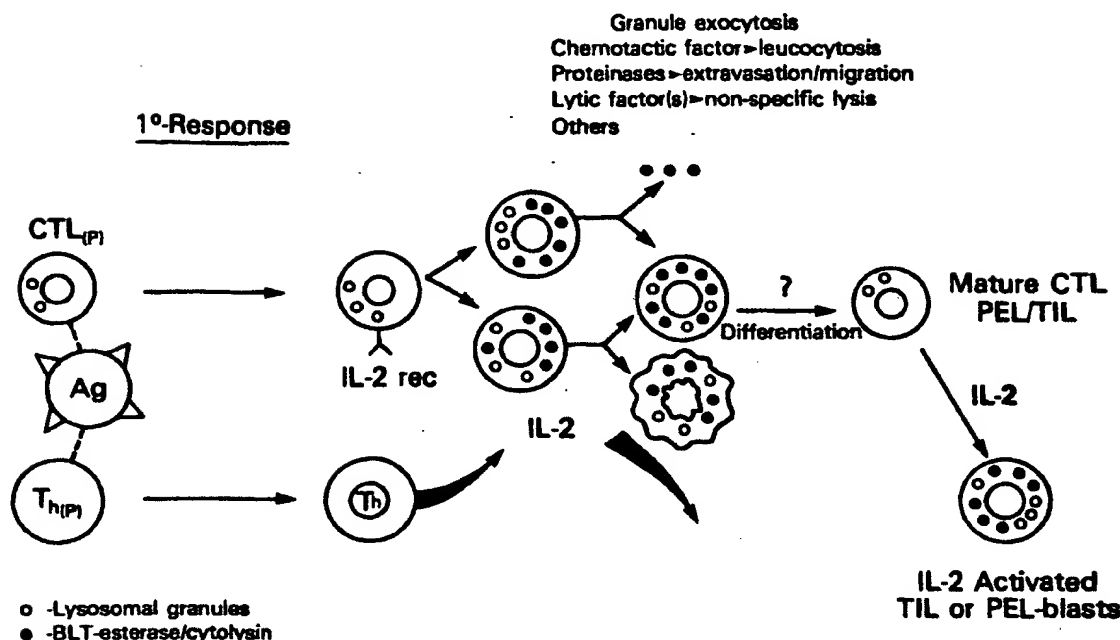


FIG. 15. The function of granules in lymphocyte activation modulation by IL-2.

and perforin mRNA (Berke, Podack and Lowry, *in preparation*). IL-2 also appears to modulate the expression of BLT-esterase and cytoplasmic granules in the course of CTL differentiation (Fig. 15), as we have found transient expression of BLT-esterase activity during primary CTL differentiation in the peritoneal cavity (Gardyn and Berke *unpublished*). Hence, granule and protease expression in CTL correlates with their stage of differentiation rather than cytotoxic activity. Granules may play a role in CTL trafficking and in response to antigen, as well as in the elimination of responding cells. The proposed mechanisms involving exocytosis of lytic granules may apply to killing induced by granule-containing effectors, such as LGLs and CTL cultured in IL-2, but not mature granule-free CTL such as PEL (Fig. 5). Thus there appear to be two distinct pathways of lymphocytotoxicity, only one of which involves secretory lytic granules.

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SCHWEIZERISCHE EIDGENOSSENSCHAFT CONFÉDÉRATION SUISSE CONFEDERAZIONE SVIZZERA

Bescheinigung

Die beiliegenden Akten stimmen überein mit den ursprünglichen technischen Unterlagen des auf der nächsten Seite bezeichneten Patentgesuches für die Schweiz und Liechtenstein.*

Attestation

Les documents ci-joints sont conformes aux pièces techniques originales de la demande de brevet pour la Suisse et le Liechtenstein* spécifiée à la page suivante.

Attestazione

Gli uniti documenti sono conformi agli atti tecnici originali della domanda di brevetto per la Svizzera e il Liechtenstein* specificata nella pagina seguente.

Bern, 15. Juni 1990

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F. HOFFMANN-LA ROCHE AG, Basel/Schweiz

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TNF-bindende Proteine

15 Tumor Nekrosis Faktor α (TNF α , auch Cachectin), auf
Grund seiner haemorrhagisch-nekrotisierenden Wirkung auf
bestimmte Tumoren entdeckt, und Lymphotoxin (TNF β) sind zwei
nah verwandte Peptidfaktoren [3] aus der Klasse der
Lymphokine/Cytokine, die im folgenden beide als TNF
20 bezeichnet werden [siehe Uebersichtsarbeiten 2 und 3]. TNF
verfügt über ein breites zelluläres Wirkungsspektrum.
Beispielsweise besitzt TNF inhibierende oder cytotoxische
Wirkung auf eine Reihe von Tumorzell-Linien [2,3],
stimuliert die Proliferation von Fibroblasten und die
25 phagozytierende/cytotoxische Aktivität von myeloischen
Zellen [4,5,6], induziert Adhäsionsmoleküle in
Endothelzellen oder übt eine inhibierende Wirkung auf
Endothel aus [7,8,9,10], inhibiert die Synthese von
spezifischen Enzymen in Adipozyten [11] und induziert die
30 Expression von Histokompatibilitätsantigenen [12]. Manche
dieser TNF-Wirkungen werden über eine Induktion von anderen
Faktoren oder durch synergistische Effekte mit anderen
Faktoren, wie beispielsweise Interferonen oder Interleukinen
erzielt [13-16].

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AB/12.9.89

TNF ist bei einer Reihe von pathologischen Zuständen, beispielsweise Schockzuständen bei Meningococcen-Sepsis [17], bei der Entwicklung von Autoimmun-Glomerulonephritis bei Mäusen [18] oder bei cerebraler Malaria bei Mäusen [19] und beim Menschen [41] involviert. Ganz allgemein scheinen die toxischen Wirkungen von Endotoxin durch TNF vermittelt zu sein [20]. Weiterhin kann TNF wie Interleukin-1 Fieber auslösen [39]. Auf Grund der pleiotropen funktionellen Eigenschaften von TNF kann man annehmen, dass TNF in Wechselwirkung mit anderen Cytokinen bei einer ganzen Reihe weiterer pathologischer Zustände als Mediator von Immunantwort, Entzündung oder anderen Prozessen beteiligt ist.

Diese biologischen Effekte werden durch TNF über spezifische Rezeptoren vermittelt, wobei nach heutigem Wissensstand sowohl TNF α wie TNF β an die gleichen Rezeptoren binden [21]. Verschiedene Zelltypen unterscheiden sich in der Anzahl von TNF-Rezeptoren [22,23,24]. Solche ganz allgemein gesprochen TNF-bindenden Proteine (TNF-BP) wurden durch kovalente Bindung an radioaktiv markiertes TNF nachgewiesen [24-29], wobei die folgenden scheinbaren Molekulargewichte der erhaltenen TNF/TNF-BP-Komplexe ermittelt wurden: 95/100 kD und 75 kD [24], 95 kD und 75 kD [25], 138 kD, 90 kD, 75 kD und 54 kD [26], 100 \pm 5 kD [27], 97 kD und 70 kD [28] und 145 kD [29]. Mittels anti-TNF-Antikörper-Immunoaffinitätschromatographie und präparativer SDS-Polyacrylamidgelelektrophorese (SDS-PAGE) konnte ein solcher TNF/TNF-BP-Komplex isoliert werden [27]. Die reduktive Spaltung dieses Komplexes und anschliessende SDS-PAGE-Analyse ergab mehrere Banden, die allerdings nicht auf TNF-Bindeaktivität getestet wurden. Da die spezifischen Bedingungen, die zu der Spaltung des Komplexes verwendet werden müssen, zur Inaktivierung des Bindeproteins führen [31], ist letzteres auch nicht möglich gewesen. Die Anreicherung von löslichen TNF-BP aus dem humanen Serum oder Urin mittels Ionenaustauscher-Chromatographie und

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- 3 -

Gelfiltration (Molekulargewichte im Bereich von 50 kD) wurde von Olssen et al. beschrieben [30].

Brockhaus et al. [32] erhielten durch
5 TNF α -Ligandenaffinitätschromatographie und HPLC aus
Membranextrakten von HL60-Zellen eine angereicherte
TNF-BP-Präparation, die wiederum als Antigenpräparation zur
Herstellung von monoklonalen Antikörpern gegen TNF-BP
verwendet wurde. Unter Verwendung eines solchen
10 immobilisierten Antikörpers (Immunaффinitätschromatogra-
phie) wurde mittels TNF α -Ligandenaffinitäts-
chromatographie und HPLC von Loetscher und Brockhaus [31]
aus einem Membranextrakt von humaner Placenta eine
angereicherte Präparation von TNF-BP erhalten, die in der
15 SDS-PAGE-Analyse eine starke breite Bande bei 35 kD, eine
schwache Bande bei etwa 40 kD und eine sehr schwache Bande
im Bereich zwischen 55 kD und 60 kD ergab. Im Übrigen zeigte
das Gel im Bereich von 33 kD bis 40 kD einen
Proteinhintergrundschmier. Die Bedeutung der so erhaltenen
20 Proteinbanden war jedoch im Hinblick auf die Heterogenität
des verwendeten Ausgangsmaterials (Placenta-Gewebe;
vereinigt Material aus mehreren Placenten) nicht klar.

Gegenstand der vorliegenden Erfindung sind nichtlösliche
25 Proteine und lösliche oder nichtlösliche Fragmente davon,
die TNF binden (TNF-BP), in homogener Form, sowie deren
physiologisch verträgliche Salze. Bevorzugt sind solche
Proteine, die gemäss SDS-PAGE unter nicht reduzierenden
Bedingungen durch scheinbare Molekulargewichte von etwa 55
30 kD, 51 kD, 38 kD, 36 kD und 34 kD bzw. 75 kD und 65 kD
charakterisiert sind, insbesondere solche mit etwa 55 kD und
75 kD. Weiterhin bevorzugt sind solche Proteine, die durch
wenigstens eine der beiden Aminosäureteilsequenzen
gekennzeichnet sind:

35 Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-
Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile

(I)

Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly

(II)

- 5 wobei X für einen Aminosäurerest steht, der nicht eindeutig bestimmt werden konnte.

Im Stand der Technik sind bereits TNF-BP durch eine N-terminale Teilsequenz charakterisiert worden [Europäische Patentanmeldung mit der Publikations-Nr. 308 378], wobei sich diese Sequenz von der erfindungsgemässen N-terminalen Teilsequenz gemäss Formel (I) unterscheidet. Im übrigen handelt es sich aber bei den im Stand der Technik beschriebenen TNF-Bindeproteinen um aus dem Urin isolierte, lösliche, d.h. nicht membrangebundene, TNF-BP und nicht um membrangebundene, d.h. unlösliche, TNF-BP.

Gegenstand der vorliegenden Anmeldung sind auch Verfahren zur Isolierung der erfindungsgemässen TNF-BP. Diese Verfahren sind dadurch charakterisiert, dass man im wesentlichen die folgenden Reinigungsschritte nacheinander ausführt: Herstellung eines Zell- oder Gewebeextraktes, Immunaффinitätschromatographie und/oder ein- oder mehrfache Ligandenaффinitätschromatographie, hochauflösende Flüssigkeitschromatographie (HPLC) und präparative SDS-Polyacrylamidgelelektrophorese (SDS-PAGE). Die Kombination der aus dem Stand der Technik bekannten einzelnen Reinigungsschritte ist für den Erfolg des erfindungsgemässen Verfahrens essentiell, wobei einzelne Schritte im Rahmen der zu lösenden Aufgabe modifiziert/verbessert wurden. So wurde beispielsweise der ursprünglich für die Anreicherung von TNF-BP aus humaner Placenta [31] verwendete kombinierte Immunaффinitätschromatographie/TNF α -Ligandenaффinitätschromatographie-Schritt dadurch abgeändert, dass eine BSA-Sepharose 4B-Vorsäule verwendet wurde. Diese Vorsäule wurde zum Auftrag des Zell- oder Membranextraktes in Reihe mit der Immunaффinitätssäule und gefolgt von der Ligandenaффinitätssäule geschaltet. Nach

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Auftrag des Extraktes wurden die beiden zuletztgenannten Säulen abgekoppelt, jede für sich eluiert und die TNF-BP-aktiven Fraktionen wurden nochmals über eine Ligandenaffinitätssäule gereinigt. Erfindungswesentlich für die Durchführung des Umkehrphasen-HPLC-Schrittes ist die Verwendung eines Detergens-haltigen Lösungsmittelgemisches.

Gegenstand der vorliegenden Erfindung sind auch pharmazeutische Präparate, die wenigstens eines dieser TNF-BP oder Fragmente davon, gewünschtenfalls in Verbindung mit weiteren pharmazeutisch wirksamen Substanzen und/oder nicht-toxischen, inerten, therapeutisch verträglichen Trägermaterialien enthalten.

Die vorliegende Erfindung betrifft schliesslich die Verwendung solcher TNF-BP einerseits zur Herstellung pharmazeutischer Präparate bzw. andererseits zur Behandlung von Krankheiten, bevorzugt solchen, in deren Verlauf TNF involviert ist.

20

Ausgangsmaterial für die erfindungsgemässen TNF-BP sind ganz allgemein Zellen, die solche TNF-BP in membrangebundener Form enthalten und die dem Fachmann ohne Beschränkungen allgemein zugänglich sind, wie beispielsweise HL60- [ATCC Nr. CCL 240], U 937- [ATCC Nr. CRL 1593], SW 480- [ATCC Nr. CCL 228] und HEP2-Zellen [ATCC Nr. CCL 23]. Diese Zellen können nach bekannten Methoden des Standes der Technik kultiviert werden [40]. TNF-BP können dann nach bekannten Methoden des Standes der Technik mittels geeigneter Detergenzien, beispielsweise Triton X-114, 1-O-n-Octyl- β -D-glucopyranosid (Octylglucosid), oder 3-[(3-Cholylamidopropyl)-dimethylammonio]-1-propan sulfonat (CHAPS), im besonderen mittels Triton X-100, aus den aus dem Medium abzentrifugierten und gewaschenen Zellen extrahiert werden. Zum Nachweis solcher TNF-BP können die üblicherweise verwendeten Nachweismethoden für TNF-BP, beispielsweise eine Polyethylenglykol-induzierte Fällung des ¹²⁵I-TNF/TNF-BP-

- Komplexes [27], im besonderen Filterbindungstests mit radioaktiv markiertem TNF gemäss Beispiel 1, verwendet werden. Zur Gewinnung der erfindungsgemässen TNF-BP können die generell zur Reinigung von Proteinen, insbesondere von
- 5 Membranproteinen, verwendeten Methoden des Standes der Technik, wie beispielsweise Ionenaustausch-Chromatographie, Gelfiltration, Affinitätschromatographie, HPLC und SDS-PAGE verwendet werden. Besonders bevorzugte Methoden zur Herstellung erfindungsgemässer TNF-BP sind
- 10 Affinitätschromatographie, insbesondere mit TNF- α als Liganden und Immunaффinitätschromatographie, HPLC und SDS-PAGE. Die Elution von mittels SDS-PAGE aufgetrennten TNF-BP Banden kann nach bekannten Methoden der Proteinchemie erfolgen, beispielsweise mittels Elektroelution nach
- 15 Hunkapiller et al. [34], wobei nach heutigem Stand des Wissens die dort angegebenen Elektro-Dialysezeiten generell zu verdoppeln sind. Danach noch verbleibende Spuren von SDS können dann gemäss Bosserhoff et al. [50] entfernt werden.
- 20 Die so gereinigten TNF-BP können mittels der im Stand der Technik bekannten Methoden der Peptidchemie, wie beispielsweise N-terminale Aminosäuresequenzierung oder enzymatische wie chemische Peptidspaltung charakterisiert werden. Durch enzymatische oder chemische Spaltung erhaltene
- 25 Fragmente können nach gängigen Methoden, wie beispielsweise HPLC, aufgetrennt und selbst wieder N-terminal sequenziert werden. Solche Fragmente, die selbst noch TNF binden, können mittels der obengenannten Nachweismethoden für TNF-BP identifiziert werden und sind ebenfalls Gegenstand der
- 30 vorliegenden Erfindung.

Ausgehend von der so erhältlichen Aminosäuresequenzinformation können unter Beachtung der Degeneration des genetischen Codes nach im Stand der Technik

35 bekannten Methoden geeignete Oligonukleotide hergestellt werden [51]. Mittels dieser können dann wiederum nach bekannten Methoden der Molekularbiologie [42,43] cDNA- oder

genomische DNA-Banken nach Klonen, die für TNF-BP kodierende Nukleinsäuresequenzen enthalten, abgesucht werden. DNA-Stücke solcher Sequenzen können dann wiederum nach bekannten Methoden sequenziert oder in geeigneten

5 Expressionsvektoren zur Expression in dazu passenden Wirtszellen gebracht werden. Die so erhaltenen TNF-BP können dann nach gängigen Methoden des Standes der Technik aus den Wirtszellen selbst isoliert werden. Ausserdem können mittels der Polymerase-Kettenreaktion (PCR) [49] cDNA-Fragmente

10 kloniert werden, indem von zwei auseinanderliegenden, relativ kurzen Abschnitten der Aminosäuresequenz unter Beachtung des genetischen Codes vollständig degenerierte und in ihrer Komplementarität geeignete Oligonucleotide als "Primer" eingesetzt werden, wodurch das zwischen diesen

15 beiden Sequenzen liegende Fragment identifiziert und amplifiziert wird. Die Bestimmung der Basensequenz eines derartigen Fragmentes ermöglicht eine unabhängige Bestimmung der Aminosäure-Sequenz des Proteinfragments für das es kodiert.

20

Die erfindungsgemäss erhaltenen TNF-BP können auch als Antigene zur Erzeugung von poly- und monoklonalen Antikörpern nach bekannten Methoden der Technik [44,45] oder gemäss dem in Beispiel 3 beschriebenen Verfahren verwendet

25 werden. Solche Antikörper, insbesondere monoklonale Antikörper gegen die 75 kD-TNF-BP-Spezies, sind ebenfalls Gegenstand der vorliegenden Erfindung.

Auf Grund der hohen Bindungsaffinität erfindungsgemässer

30 TNF-BP für TNF (K_d -Werte um 10^{-10} M) können diese oder Fragmente davon als Diagnostika zum Nachweis von TNF in Serum oder anderen Körperflüssigkeiten nach im Stand der Technik bekannten Methoden, beispielsweise in Festphasenbindungstests oder in Verbindung mit Anti-TNF-BP-

35 Antikörpern in sogenannten "Sandwich"-Tests, eingesetzt werden.

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Im Übrigen können erfindungsgemässe TNF-BP einerseits zur Reinigung von TNF und andererseits zum Auffinden von TNF-Agonisten so wie TNF-Antagonisten nach im Stand der Technik bekannten Verfahren verwendet werden.

5

Die erfindungsgemässen TNF-BP sowie deren physiologisch verträgliche Salze können auch zur Herstellung von pharmazeutischen Präparaten, vor allem solchen zur Behandlung von Krankheiten, bei deren Verlauf TNF involviert ist, verwendet werden. Dazu kann eine oder mehrere der genannten Verbindungen, falls wünschenswert bzw. erforderlich in Verbindung mit anderen pharmazeutisch aktiven Substanzen, mit den üblicherweise verwendeten festen oder flüssigen Trägermaterialien in bekannter Weise verarbeitet werden. Die Dosierung solcher Präparate kann unter Berücksichtigung der üblichen Kriterien in Analogie zu bereits verwendeten Präparaten ähnlicher Aktivität und Struktur erfolgen.

20 Nachdem die Erfindung vorstehend allgemein beschrieben worden ist, sollen die folgenden Beispiele Einzelheiten der Erfindung veranschaulichen, ohne dass diese dadurch in irgendeiner Weise eingeschränkt wird.

25

Beispiel 1

Nachweis von TNF-bindenden Proteinen

Die TNF-BP wurden in einem Filtrertest mit humanem radiojodiertem ¹²⁵I-TNF nachgewiesen. TNF (46,47) wurde mit Na ¹²⁵I (IMS40, Amersham, Amersham, England) und Iodo-Gen (#28600, Pierce Eurochemie, Oud-Beijerland, Niederlande) nach Fraker und Speck [48] radioaktiv markiert. Zum Nachweis der TNF-BP wurden isolierte Membranen der Zellen oder ihre solubilisierten, angereicherten und gereinigten Fraktionen auf angefeuchtete Nitrocellulose-Filter (0.45 µ, BioRad, Richmond,

- California, USA) aufgetragen. Die Filter wurden dann in Pufferlösung mit 1% entfettetem Milchpulver blockiert und anschliessend mit $5 \cdot 10^5$ cpm/ml ^{125}I -TNF α (0.3 - $1.0 \cdot 10^8$ cpm/ μg) in zwei Ansätzen mit und ohne
- 5 Beigabe von $5 \mu\text{g/ml}$ nicht-markiertem TNF α inkubiert, gewaschen und luftgetrocknet. Die gebundene Radioaktivität wurde autoradiographisch semiquantitativ nachgewiesen oder in einem γ -Counter gezählt. Die spezifische ^{125}I -TNF-Bindung wurde nach Korrektur für unspezifische
- 10 Bindung in Anwesenheit von unmarkiertem TNF im Ueberschuss ermittelt. Die spezifische TNF-Bindung im Filtertest wurde bei verschiedenen TNF-Konzentrationen gemessen und nach Scatchard analysiert [33], wobei ein K_d -Wert von $\sim 10^{-9}$ - 10^{-10}M ermittelt wurde.

15

Beispiel 2

Zellextrakte von HL-60-Zellen

- 20 HL60 Zellen [ATCC-Nr. CCL 240] wurden in einem RPMI 1640-Medium [GIBCO-Katalog Nr. 074-01800], das noch 2 g/l NaHCO_3 und 5% fötales Kälberserum enthielt in einer 5%-igen CO_2 -Atmosphäre kultiviert, zentrifugiert und mit isotonem Phosphatpuffer (PBS; 0.2 g/l KCl, 0.2 g/l
- 25 KH_2PO_4 , 8.0 g/l NaCl, 2.16 g/l $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$), der mit 5% Dimethylformamid, 10 mM Benzamidin, 100 E/ml Aprotinin, 10 μM Leupeptin, 1 μM Pepstatin, 1 mM o-Phenanthrolin, 5 mM Jodacetamid, 1 mM Phenylmethylsulfonylfluorid versetzt war (im folgenden als
- 30 PBS-M bezeichnet), gewaschen. Die gewaschenen Zellen wurden bei einer Dichte von $2.5 \cdot 10^8$ Zellen/ml in PBS-M mit Triton X-100 (Endkonzentration 1.0%) extrahiert. Der Zellextrakt wurde durch Zentrifugation geklärt ($15'000 \times \text{g}$, 1 Stunde; $100'000 \times \text{g}$, 1 Stunde).

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Beispiel 3

Herstellung von monoklonalen (TNF-BP)-Antikörpern

5 Der gemäss Beispiel 2 erhaltene Zentrifugationsüberstand wurde im Verhältnis 1:10 mit PBS verdünnt. Der verdünnte Überstand wurde bei 4°C auf eine Säule aufgetragen (Flussrate: 0.2 ml/min.), die 2 ml Affigel 10 enthielt (Bio Rad Katalog Nr. 153-6099) an das 20 mg rekombinantes humanes
10 TNF- α [Pennica, D. et al. (1984) Nature 312, 724; Shirai, T. et al. (1985) Nature 313, 803; Wang, A.M. et al. (1985) Science 228, 149] gemäss den Empfehlungen des Herstellers gekoppelt worden war. Die Säule wurde bei 4°C und einer Durchflussrate von 1 ml/min zuerst mit 20 ml PBS, das 0.1%
15 Triton X 114 enthielt und danach mit 20 ml PBS gewaschen. So angereichertes TNF-BP wurde bei 22°C und einer Flussrate von 2 ml/min mit 4 ml 100 mM Glycin, pH 2.8, 0.1% Decylmaltosid eluiert. Das Eluat wurde in einer Centricon 30 Einheit [Amicon] auf 10 μ l konzentriert.

20 10 μ l dieses Eluates wurden mit 20 μ l vollständigem Freundschens Adjuvans gemischt. 10 μ l der Emulsion wurden gemäss dem von Holmdahl, R. et al. [(1985), J. Immunol. Methods 83, 379] beschriebenen Verfahren an den Tagen 0, 7
25 und 12 in eine hintere Fusspfote einer narkotisierten Balb/c-Maus injiziert.

Am Tag 14 wurde die immunisierte Maus getötet und der popliteale Lymphknoten herausgenommen, zerkleinert und in
30 Iscove's Medium (IMEM, GIBCO Katalog Nr. 074-2200), das 2 g/l NaHCO₃ enthielt, durch wiederholtes Pipettieren suspendiert. Gemäss einem modifizierten Verfahren von De St. Groth und Scheidegger [J. Immunol. Methods (1980), 35, 1] wurden 5×10^7 Zellen des Lymphknotens mit 5×10^7 PAI Maus-
35 Myelomazellen (J.W. Stocker et al., Research Disclosure, 217, Mai 1982, 155-157), die sich in logarithmischem Wachstum befanden, fusioniert. Die Zellen wurden gemischt,

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durch Zentrifugation gesammelt und durch leichtes Schütteln in 2 ml 50% (v/v) Polyethylenglycol in IMEM bei Raumtemperatur resuspendiert und durch langsame Zugabe von 10 ml IMEM während 10 Minuten vorsichtigen Schüttelns verdünnt. Die Zellen wurden durch Zentrifugation gesammelt und in 200 ml vollständigem Medium [IMEM + 20% fötales Kälberserum, Glutamin (2.0 mM), 2-Mercaptoethanol (100 µM), 100 µM Hypoxanthine, 0.4 µM Aminopterin und 16 µM Thymidine (HAT)] resuspendiert. Die Suspension wurde auf 10 Gewebekulturschalen, die jeweils 96 Vertiefungen enthielten, verteilt und ohne Wechsel des Mediums bei 37°C in einer Atmosphäre von 5% CO₂ und einer Luftfeuchtigkeit von 98% 11 Tage lang inkubiert.

Die Antikörper zeichnen sich aus durch ihre inhibierende Wirkung auf die TNF-Bindung an HL60-Zellen sowie durch ihre Bindung an Antigen im Filtertest gemäss Beispiel 1.

Zum Nachweis der biologischen Aktivität von anti-(TNF-BP)-Antikörpern wurde folgendermassen verfahren. 5x10⁶ HL60-Zellen oder U937-Zellen wurden in vollständigem RPMI 1640 Medium zusammen mit affinitätsgereinigten monoklonalen anti-(TNF-BP)-Antikörpern oder Kontrollantikörpern (d.h. solchen, die nicht gegen TNF-BP gerichtet sind) in einem Konzentrationsbereich von 1 ng/ml bis 10 µg/ml inkubiert. Nach einer Stunde Inkubation bei 37°C wurden die Zellen durch Zentrifugation gesammelt und mit 4.5 ml PBS bei 0°C gewaschen. Sie wurden in 1 ml vollständigem RPMI 1640 Medium (Beispiel 2), das zusätzlich 0.1% Natriumazid und ¹²⁵I-TNF (10⁶ cpm/ml) (s.o.) enthielt, resuspendiert. Die spezifische Radioaktivität betrug 700 Ci/mmol. Die Zellen wurden 2 Stunden bei 4°C inkubiert, gesammelt und 4 mal mit 4.5 ml PBS das 1% BSA und 0.001% Triton X 100 (Fluka) enthielt bei 0°C gewaschen. Die an die Zellen gebundene Radioaktivität wurde in einem γ-Scintillationszähler gemessen. In einem vergleichbaren Experiment wurde die zellgebundene

Radioaktivität von Zellen, die nicht mit anti-(TNF-BP)-Antikörpern behandelt worden waren, bestimmt (ungefähr 10 000 cpm/5x10⁶ Zellen).

5

Beispiel 4Affinitätschromatographie

Für die weitere Reinigung wurden jeweils ein gemäss
10 Beispiel 3 erhaltener monoklonaler anti-(TNF-BP)-Antikörper (2,8 mg/ml Gel), TNF α (3,0 mg/ml Gel) und Rinderserumalbumin (BSA, 8,5 mg/ml Gel) gemäss den Vorschriften des Herstellers kovalent an CNBr-aktivierte Sepharose 4B (Pharmacia, Uppsala, Schweden) gekoppelt. Der
15 gemäss Beispiel 2 erhaltene Zellextrakt wurde über die so hergestellten und in der folgenden Reihenfolge hintereinandergeschalteten Säulen geleitet:
BSA-Sepharose-Vorsäule, Immunaффinitätssäule [Anti-(TNF-BP)-Antikörper], TNF α -Ligand-Aффinitätssäule.
20 Nach vollständigem Auftrag wurden die beiden letztgenannten Säulen abgetrennt und einzeln für sich mit je 100 ml der folgenden Pufferlösungen gewaschen: (1) PBS, 1.0% Triton X-100, 10 mM Benzamidin, 100 E/ml Aprotinin; (2) PBS, 0.1% Triton X-100, 0.5M NaCl, 10 mM ATP, 10 mM Benzamidin,
25 100 E/ml Aprotinin; und (3) PBS, 0.1% Triton X-100, 10 mM Benzamidin, 100 E/ml Aprotinin. Sowohl die Immun- als auch die TNF α -Ligand-Aффinitätssäule wurden dann mit 100 mM Glycin pH 2.5, 100 mM NaCl, 0.2% Dacylmaltoside, 10 mM Benzamidin, 100 E/ml Aprotinin jede für sich eluiert. Die im
30 Filtertest gemäss Beispiel 1 aktiven Fraktionen jeder Säule wurden danach jeweils vereint und mit 1M Tris pH 8.0 neutralisiert.

Die so vereinten TNF-BP-aktiven Fraktionen der
35 Immun-Aффinitätschromatographie einerseits und der TNF α -Ligand-Aффinitätschromatographie andererseits wurden zur weiteren Reinigung nochmals auf je eine kleine

- TNFr-Ligand-Affinitätssäule aufgetragen. Danach wurden diese beiden Säulen mit je 40 ml von (1) PBS, 1.0% Triton X-100, 10 mM Benzamidin, 100 E/ml Aprotinin, (2) PBS, 0.1% Triton X-100, 0.5M NaCl, 10 mM ATP, 10mM Benzamidin, 5 100 E/ml Aprotinin, (3) PBS, 0.1% Triton X-100, (4) 50 mM Tris pH 7.5, 150 mM NaCl, 1.0% NP-40, 1.0% Desoxycholat, 0.1% SDS, (5) PBS, 0.2% Decylmaltosid gewaschen. Anschliessend wurden die Säulen mit 100 mM Glycin pH 2.5, 100 mM NaCl, 0.2% Decylmaltosid eluiert. Fraktionen von 10 0.5 ml von jeder Säule wurden für sich gesammelt und die gemäss Filtertest (Beispiel 1) aktiven Fraktionen von jeder Säule jeweils für sich vereint und in einer Centricon-Einheit (Amicon, Molekulargewichts-Ausschluss 10'000) aufkonzentriert.

15

Beispiel 5Auftrennung mittels HPLC

- 20 Die gemäss Beispiel 4 erhaltenen aktiven Fraktionen wurden gemäss ihrer unterschiedlichen Herkunft (Immun- bzw. Ligand-Affinitätschromatographie) jeweils für sich auf C1/C8 Umkehrphasen-HPLC-Säulen (ProRPC, Pharmacia, 5x20 mm), die mit 0.1% Trifluoressigsäure, 0.1% Octylglucosid equilibriert 25 worden waren, aufgetragen. Die Säulen wurden dann mit einem linearen Acetonitril-Gradienten (0-80%) im gleichen Puffer bei einem Fluss von 0.5 ml/min eluiert. Fraktionen von 1.0 ml wurden von jeder Säule gesammelt und die aktiven Fraktionen von jeder Säule für sich vereint (Nachweis gemäss 30 Beispiel 1).

Beispiel 6Auftrennung mittels SDS-PAGE

35

Die gemäss Beispiel 5 erhaltenen und gemäss Filtertest (Beispiel 1) aktiven Fraktionen wurden durch SDS-PAGE gemäss

[34] weiter aufgetrennt. Dazu wurden die Proben in SDS-Probenpuffer während 3 Minuten auf 95°C erhitzt und anschliessend auf einem 12% Acrylamid-Trenngel mit einem 5%igen Sammelgel elektrophoretisch aufgetrennt. Als Referenz zur Bestimmung der scheinbaren Molekulargewichte auf dem SDS-PAGE Gel wurden die folgenden Eichproteine verwendet: Phosphorylase B (97.4 kD), BSA (66.2 kD), Ovalbumin (42.7 kD), Carboanhydrase (31.0 kD), Soya Trypsin-Inhibitor (21.5 kD) und Lysozym (14.4 kD).

10 Unter den genannten Bedingungen wurden für Proben, die gemäss Beispiel 4 durch TNF- α -Ligandenaffinitätschromatographie von Immunaффinitätschromatographieeluat erhalten und durch HPLC gemäss Beispiel 5 weiter aufgetrennt worden waren, zwei Banden von 55 kD und 51 kD sowie drei schwächere Banden von 38 kD, 36 kD und 34 kD erhalten. Diese Banden wurden in einem Mini Trans Blot System (BioRad, Richmond, California, USA) elektrophoretisch während 1 Stunde bei 100 V in 25 mM Tris, 192 mM Glycin, 20% Methanol auf eine PVDF-Membran (Immobilon, Millipore, Bedford, Mass. USA) transferiert. Danach wurde die PVDF-Membran entweder mit 0.15% Serva-Blau (Serva, Heidelberg, BRD) in Methanol/Wasser/Eisessig (50/40/10 Volumenteile) auf Protein gefärbt oder mit entfettetem Milchpulver blockiert und 25 anschliessend zum Nachweis von Banden mit TNF-BP-Aktivität mit ^{125}I -TNF gemäss den in Beispiel 1 beschriebenen Filtertestbedingungen inkubiert. Dabei zeigte sich, dass alle in der Proteinfärbung zur Darstellung gelangten Banden spezifisch TNF α banden. Alle diese Banden banden im 30 Western Blot nach Towbin et al. [38] auch den gemäss Beispiel 3 hergestellten monoklonalen Antikörper. Dabei wurde ein gemäss dem in Beispiel 1 beschriebenen Verfahren mit Na ^{125}I radioaktiv markierter, affinitätsgereinigter (Mausimmunglobulin-Sepharose-4B-Affinitätssäule) 35 Kaninchen-anti-Maus-Immunglobulin-Antikörper zum autoradiographischen Nachweis dieses Antikörpers eingesetzt.

Proben, die gemäss Beispiel 4 durch zweimalige
TNF- α -Ligandenaffinitätschromatographie des Durchlaufs der
Immunaффinitätschromatographie erhalten und durch HPLC
gemäss Beispiel 5 weiter aufgetrennt worden waren, zeigten
5 unter den oben spezifizierten SDS-PAGE- und
Blottransfer-Bedingungen zwei zusätzliche Banden von 75 kD
und 65 kD, die beide im Filtertest (Beispiel 1) spezifisch
TNF banden. Im Western Blot gemäss Towbin et al. (s.o.)
reagierten die Proteine dieser beiden Banden nicht mit dem
10 gemäss Beispiel 3 hergestellten Antikörper. Sie reagierten
allerdings mit einem monoklonalen Antikörper, der ausgehend
von der 75 kD-Bande erzeugt worden war.

Beispiel 7

15

Aminosäuresequenzanalyse

Zur Aminosäuresequenzanalyse wurden die gemäss
Beispiel 5 erhaltenen und gemäss Filtertest (Beispiel 1)
20 aktiven Fraktionen mittels der in Beispiel 6 beschriebenen,
nun jedoch reduzierenden, SDS-PAGE Bedingungen
(SDS-Probenpuffer mit 125 mM Dithiothreitol) aufgetrennt. Es
wurden die gleichen Banden wie gemäss Beispiel 6 gefunden,
die allerdings auf Grund der reduzierenden Bedingungen der
25 SDS-PAGE im Vergleich zu Beispiel 6 alle um etwa 1-2 kD
höhere Molekulargewichte zeigten. Diese Banden wurden dann
gemäss Beispiel 6 auf PVDF-Membranen übertragen und mit
0.15% Serva-Blau in Methanol/Wasser/ Eisessig (50/40/10
Volumenteile) während 1 Minute gefärbt, mit
30 Methanol/Wasser/Eisessig (45/48/7 Volumenteile) entfärbt,
mit Wasser gespült, luftgetrocknet und danach
ausgeschnitten. Bei sämtlichen Schritten wurden zur
Vermeidung von N-terminaler Blockierung die von Hunkapiller
[34] angegebenen Bedingungen eingehalten. So vorbereitete
35 Proben wurden dann in einem automatisierten
Gasphasen-Mikrosequenzier-Gerät (Applied Biosystems Modell
470A, ABI, Foster City, Calif., USA) mit einem on-line

nachgeschalteten automatisierten HPLC

PTH-Aminosäureanalysator (Applied Biosystems Modell 120, ABI s.o.) sequenziert, wobei die folgenden Aminosäuresequenzen bestimmt wurden:

5

1., Für die 55 kD-Bande (gemäss nichtreduzierender SDS-PAGE):
Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-
Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile,
wobei X für einen Aminosäurerest steht, der nicht
bestimmt werden konnte.

10

2., Für die 51 kD und die 38 kD-Banden (gemäss
nichtreduzierender SDS-PAGE):
Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu

15

3., Für die 65 kD-Bande (gemäss nichtreduzierender SDS-PAGE):
Bei der N-terminalen Sequenzierung der 65 kD Bande
wurden bis zum 15. Rest ohne Unterbrechung zwei
parallele Sequenzen ermittelt. Da eine der beiden
Sequenzen einer Teilsequenz des Ubiquitins [36,37]
entsprach, wurde für die 65 kD-Bande die folgende
Sequenz abgeleitet:

20

Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly

25

wobei X für einen Aminosäurerest steht, der nicht
bestimmt werden konnte.

Beispiel 8

30

Bestimmung von Basen-Sequenzen von komplementärer DNA (cDNA)

Ausgehend von der Aminosäuresequenz gemäss Formel I
wurden unter Berücksichtigung des genetischen Codes zu den
Aminosäureresten 2-7 und 17-23 entsprechende, vollständig
degenerierte Oligonucleotide in geeigneter Komplementarität
synthetisiert ("sense" and "antisense" Oligonucleotide).

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Totale zelluläre RNA wurde aus HL60-Zellen isoliert [42, 43], und der erste cDNA-Strang durch Oligo-dT-Priming oder durch Priming mit dem "antisense" Oligonucleotid mittels eines cDNA-Synthese-Kits (RPN 1256, 5 Amersham, Amersham, England) gemäss der Anleitung des Herstellers synthetisiert. Dieser cDNA-Strang und die beiden synthetisierten degenerierten Oligonucleotide wurden in einer Polymerase-Kettenreaktion (PCR Perkin Elmer Cetus, Norwalk, CT, USA gemäss Anleitung des Herstellers) dazu 10 verwendet, die für die Aminosäure-Reste 8-16 (Formel I) codierende Basensequenz als cDNA-Fragment zu synthetisieren. Die Basensequenz dieses cDNA-Fragmentes lautet: 5'-AGGGAG-AAGAGAGATAGTGTGTGTC-3'.

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Patentansprüche

1. Nichtlösliche Proteine und lösliche oder nicht-
lösliche Fragmente davon, die TNF binden, in homogener Form,
5 sowie deren physiologisch verträgliche Salze.

2. Proteine gemäss Anspruch 1, die durch
Molekulargewichte gemäss SDS-PAGE unter nichtreduzierenden
Bedingungen von etwa 55 kD und 75 kD charakterisiert sind.
10

3. Proteine gemäss einem der Ansprüche 1 und 2, die
wenigstens eine der folgenden Aminosäuresequenzen enthalten:

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-
15 Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile

(I).

bzw.

20 Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-
Gly,

(II)

wobei X für einen nicht bestimmten Aminosäurerest steht.

4. Ein Verfahren zur Isolierung eines Proteins gemäss
25 einem der Ansprüche 1-3, dadurch gekennzeichnet, dass man im
wesentlichen die folgenden Reinigungsschritte nacheinander
ausführt: Herstellung eines Zellextraktes,
Immunaффinitätschromatographie und/oder ein- oder mehrfache
Ligandaффinitätschromatographie, HPLC und präparative
30 SDS-PAGE.

5. Pharmazeutische Präparate, dadurch gekennzeichnet,
dass sie eine oder mehrere Verbindung(en) gemäss einem der
Ansprüche 1-3, gewünschtenfalls in Kombination mit weiteren
35 pharmazeutisch wirksamen Substanzen und/oder nicht-toxischen,
inerten, therapeutisch verträglichen Trägermaterialien
enthalten.

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6. Verwendung einer Verbindung gemäss einem der Ansprüche 1-3 zur Herstellung pharmazeutischer Präparate bzw. zur Behandlung von Krankheiten, bevorzugt solchen, bei denen TNF involviert ist.

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7. Gegen ein Protein gemäss Ansprüche 1-3 gerichtete Antikörper.

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SCHWEIZERISCHE EIDGENOSSENSCHAFT CONFÉDÉRATION SUISSE CONFEDERAZIONE SVIZZERA

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Die beiliegenden Akten stimmen überein mit den ursprünglichen technischen Unterlagen des auf der nächsten Seite bezeichneten Patentgesuches für die Schweiz und Liechtenstein.*

Attestation

Les documents ci-joints sont conformes aux pièces techniques originales de la demande de brevet pour la Suisse et le Liechtenstein* spécifiée à la page suivante.

Attestazione

Gli uniti documenti sono conformi agli atti tecnici originali della domanda di brevetto per la Svizzera e il Liechtenstein* specificate nella pagina seguente.

Bern, 15. Juni 1990

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Voraussichtliche Klasse(n): C07K/A01N/C07H/C12N Patentgesuch Nr. 00 746/90-4 ✓

Patent-
bewerber: F. Hoffmann-La Roche AG
4002 Grenzacherstrasse 124
Basel
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Schweiz

Titel: TNF-bindende Proteine.

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TNF-bindende Proteine

- 15 Tumor Nekrosis Faktor α (TNF α , auch Cachectin), auf
Grund seiner haemorrhagisch-nekrotisierenden Wirkung auf
bestimmte Tumoren entdeckt, und Lymphotoxin (TNF β) sind zwei
nahe verwandte Peptidfaktoren [3] aus der Klasse der Lympho-
kine/Cytokine, die im folgenden beide als TNF bezeichnet
20 werden [siehe Uebersichtsarbeiten 2 und 3]. TNF verfügt über
ein breites zelluläres Wirkungsspektrum. Beispielsweise
besitzt TNF inhibierende oder cytotoxische Wirkung auf eine
Reihe von Tumorzelllinien [2,3], stimuliert die Prolifera-
tion von Fibroblasten und die phagozytierende/cytotoxische
25 Aktivität von myeloischen Zellen [4,5,6], induziert
Adhäsionsmoleküle in Endothelzellen oder übt eine inhibie-
rende Wirkung auf Endothel aus [7,8,9,10], inhibiert die
Synthese von spezifischen Enzymen in Adipozyten [11] und
induziert die Expression von Histokompatibilitätsantigenen
30 [12]. Manche dieser TNF-Wirkungen werden über eine Induktion
von anderen Faktoren oder durch synergistische Effekte mit
anderen Faktoren, wie beispielsweise Interferonen oder
Interleukinen erzielt [13-16].

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TNF ist bei einer Reihe von pathologischen Zuständen, beispielsweise Schockzuständen bei Meningococcen-Sepsis [17], bei der Entwicklung von Autoimmun-Glomerulonephritis bei Mäusen [18] oder bei cerebraler Malaria bei Mäusen [19] und beim Menschen [41] involviert. Ganz allgemein scheinen die toxischen Wirkungen von Endotoxin durch TNF vermittelt zu sein [20]. Weiterhin kann TNF wie Interleukin-1 Fieber auslösen [39]. Auf Grund der pleiotropen funktionellen Eigenschaften von TNF kann man annehmen, dass TNF in Wechselwirkung mit anderen Cytokinen bei einer ganzen Reihe weiterer pathologischer Zustände als Mediator von Immunantwort, Entzündung oder anderen Prozessen beteiligt ist.

Diese biologischen Effekte werden durch TNF über spezifische Rezeptoren vermittelt, wobei nach heutigem Wissensstand sowohl TNF α wie TNF β an die gleichen Rezeptoren binden [21]. Verschiedene Zelltypen unterscheiden sich in der Anzahl von TNF-Rezeptoren [22,23,24]. Solche ganz allgemein gesprochen TNF-bindenden Proteine (TNF-BP) wurden durch kovalente Bindung an radioaktiv markiertes TNF nachgewiesen [24-29], wobei die folgenden scheinbaren Molekulargewichte der erhaltenen TNF/TNF-BP-Komplexe ermittelt wurden: 95/100 kD und 75 kD [24], 95 kD und 75 kD [25], 138 kD, 90 kD, 75 kD und 54 kD [26], 100 \pm 5 kD [27], 97 kD und 70 kD [28] und 145 kD [29]. Mittels anti-TNF-Antikörper-Immunoaffinitätschromatographie und präparativer SDS-Polyacrylamidgelelektrophorese (SDS-PAGE) konnte ein solcher TNF/TNF-BP-Komplex isoliert werden [27]. Die reduktive Spaltung dieses Komplexes und anschliessende SDS-PAGE-Analyse ergab mehrere Banden, die allerdings nicht auf TNF-Bindeaktivität getestet wurden. Da die spezifischen Bedingungen, die zu der Spaltung des Komplexes verwendet werden müssen, zur Inaktivierung des Bindeproteins führen [31], ist letzteres auch nicht möglich gewesen. Die Anreicherung von löslichen TNF-BP aus dem humanen Serum oder Urin mittels Ionenaustauscher-Chromatographie und Gelfiltration (Molekulargewichte im Bereich von 50 kD) wurde von

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Olsson et al. beschrieben [30].

5 Brockhaus et al. [32] erhielten durch TNF α -Liganden-
affinitätschromatographie und HPLC aus Membranextrakten von
HL60-Zellen eine angereicherte TNF-BP-Präparation, die
wiederum als Antigenpräparation zur Herstellung von mono-
klonalen Antikörpern gegen TNF-BP verwendet wurde. Unter
Verwendung eines solchen immobilisierten Antikörpers (Immun-
affinitätschromatographie) wurde mittels TNF α -Liganden-
10 affinitätschromatographie und HPLC von Loetscher und
Brockhaus [31] aus einem Extrakt von humaner Placenta eine
angereicherte Präparation von TNF-BP erhalten, die in der
SDS-PAGE-Analyse eine starke breite Bande bei 35 kD, eine
schwache Bande bei etwa 40 kD und eine sehr schwache Bande
15 im Bereich zwischen 55 kD und 60 kD ergab. Im übrigen zeigte
das Gel im Bereich von 33 kD bis 40 kD einen Protein-
hintergrundschmier. Die Bedeutung der so erhaltenen Protein-
banden war jedoch im Hinblick auf die Heterogenität des
verwendeten Ausgangsmaterials (Placenta-Gewebe; vereinigt
20 Material aus mehreren Placenten) nicht klar.

Gegenstand der vorliegenden Erfindung sind nichtlösliche
Proteine und lösliche oder nichtlösliche Fragmente davon,
die TNF binden (TNF-BP), in homogener Form, sowie deren
25 physiologisch verträgliche Salze. Bevorzugt sind solche
Proteine, die gemäss SDS-PAGE unter nicht reduzierenden
Bedingungen durch scheinbare Molekulargewichte von etwa
55 kD, 51 kD, 38 kD, 36 kD und 34 kD bzw. 75 kD und 65 kD
charakterisiert sind, insbesondere solche mit etwa 55 kD und
30 75 kD. Weiterhin bevorzugt sind solche Proteine, die durch
wenigstens eine der folgenden Aminosäureteilsequenzen
gekennzeichnet sind:

(IA) Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-
35 Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-
Ile

- (IB) Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys
- (IIA) Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu
- 5 (IIB) Val-Phe-Cys-Thr
- (IIC) Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala
- (IID) Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys
- 10 (IIE) Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu
- (IIF) Leu-Cys-Ala-Pro
- (IIG) Val-Pro-His-Leu-Pro-Ala-Asp
- (IIH) Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro
- 15 wobei X für einen Aminosäurerest steht, der nicht eindeutig bestimmt werden konnte.

Im Stand der Technik sind bereits TNF-BP durch eine N-terminale Teilsequenz charakterisiert worden [Europäische Patentanmeldung mit der Publikations-Nr. 308 378], wobei

20 sich diese Sequenz von der erfindungsgemässen N-terminalen Teilsequenz gemäss Formel (IA) unterscheidet. Im übrigen handelt es sich aber bei den im Stand der Technik beschriebenen TNF-Bindeproteinen um aus dem Urin isolierte,

25 lösliche, d.h. nicht membrangebundene, TNF-BP und nicht um membrangebundene, d.h. unlösliche, TNF-BP.

Gegenstand der vorliegenden Anmeldung sind auch Verfahren zur Isolierung der erfindungsgemässen TNF-BP. Diese

30 Verfahren sind dadurch charakterisiert, dass man im wesentlichen die folgenden Reinigungsschritte nacheinander ausführt: Herstellung eines Zell- oder Gewebeextraktes, Immunoaffinitätschromatographie und/oder ein- oder mehrfache

Ligandenaffinitätschromatographie, hochauflösende Flüssigkeitschromatographie (HPLC) und präparative SDS-Polyacrylamidgelelektrophorese (SDS-PAGE). Die Kombination der aus

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dem Stand der Technik bekannten einzelnen Reinigungsschritte ist für den Erfolg des erfindungsgemässen Verfahrens essentiell, wobei einzelne Schritte im Rahmen der zu lösenden Aufgabe modifiziert und verbessert wurden. So wurde
5 beispielsweise der ursprünglich für die Anreicherung von TNF-BP aus humaner Placenta [31] verwendete kombinierte Immunaффinitätschromatographie/TNF α -Ligandenaffinitätschromatographie-Schritt dadurch abgeändert, dass eine BSA-Sepharose 4B-Vorsäule verwendet wurde. Diese Vorsäule wurde zum
10 Auftrag des Zell- oder Membranextraktes in Reihe mit der Immunaффinitätssäule und gefolgt von der Ligandenaffinitätssäule geschaltet. Nach Auftrag des Extraktes wurden die beiden zuletztgenannten Säulen abgekoppelt, jede für sich eluiert und die TNF-BP-aktiven Fraktionen wurden nochmals
15 über eine Ligandenaffinitätssäule gereinigt. Erfindungswesentlich für die Durchführung des Umkehrphasen-HPLC-Schrittes ist die Verwendung eines Detergens-haltigen Lösungsmittelgemisches.

20 Ferner ist auch ein technisches Verfahren zum Erzielen hoher Zelldichten von Säugerzellen, aus denen TNF-BP isoliert werden können, Gegenstand der vorliegenden Erfindung. Ein solches Verfahren zeichnet sich dadurch aus, dass ein Medium, welches für die spezifischen Wachstumserfordernisse
25 der verwendeten Zelllinie entwickelt wurde, in Verbindung mit einer wie z.B. im Detail in Beispiel 2 beschriebenen Perfusionsapparatur verwendet wird. Mittels eines solchen Verfahrens lassen sich beispielsweise für HL-60-Zellen bis zu mehr als 20-fach höhere Zelldichten als üblich erzielen.

30 Zusätzlich dazu betrifft die vorliegende Erfindung auch DNA-Sequenzen, die für Proteine und lösliche oder nicht-lösliche Fragmente davon, die TNF binden, kodieren. Bevorzugt sind DNA-Sequenzen, welche für ein solches Protein mit
35 einem scheinbaren Molekulargewicht von etwa 55 kD kodieren, wobei die in Abbildung 1 dargestellte Sequenz besonders bevorzugt ist, wie Sequenzen, die für nichtlösliche wie

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lösliche Fragmente von solchen Proteinen kodieren. Eine besonders bevorzugte DNA-Sequenz, die für ein nicht-lösliches Protein-Fragment kodiert, reicht von Nukleotid -185 bis 1122 der in Abbildung 1 gezeigten Sequenz. Besonders bevorzugte DNA-Sequenzen, die für lösliche Protein-Fragmente kodieren, sind solche, die von Nukleotid -185 bis 633 bzw. von Nukleotid -14 bis 633 der in Abbildung 1 gezeigten Sequenz reichen. Die vorliegende Erfindung betrifft natürlich auch die von solchen DNA-Sequenzen kodierten rekombinanten Proteine. Selbstverständlich sind dabei auch solche Proteine umfasst, in deren Aminosäuresequenzen, beispielsweise mittels gezielter Mutagenese, Aminosäuren so ausgetauscht worden sind, dass dadurch die Aktivität der TNF-BP oder deren Fragmente, nämlich die Bindung von TNF oder die Wechselwirkung mit anderen, an der Signalübertragung beteiligten Membrankomponenten, in einer gewünschten Art verändert oder erhalten wurden. Aminosäureaustausche in Proteinen und Peptiden, die im allgemeinen die Aktivität solcher Moleküle nicht verändern, sind im Stand der Technik bekannt und beispielsweise von H. Neurath und R.L. Hill in "The Proteins" (Academic Press, New York, 1979, siehe besonders Figur 6, Seite 14) beschrieben. Die am häufigsten vorkommenden Austausche sind: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly, sowie solche in umgekehrter Weise. Die vorliegende Erfindung betrifft ferner Vektoren, die erfindungsgemässe DNA-Sequenzen enthalten und zur Transformation von geeigneten pro- wie eukaryotischen Wirtssystemen geeignet sind, wobei solche Vektoren bevorzugt sind, deren Verwendung zur Expression der von den erfindungsgemässen DNA-Sequenzen kodierten Proteine führt. Schliesslich betrifft die vorliegende Erfindung auch noch mit solchen Vektoren transformierte pro- wie eukaryotische Wirtssysteme, wie Verfahren zur Herstellung von erfindungsgemässen rekombinanten Verbindungen durch Kultivierung

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solcher Wirtssysteme und anschliessende Isolierung dieser Verbindungen aus den Wirtssystemen selbst oder deren Kulturüberständen.

5 Gegenstand der vorliegenden Erfindung sind auch pharmazeutische Präparate, die wenigstens eines dieser TNF-BP oder Fragmente davon, gewünschtenfalls in Verbindung mit weiteren pharmazeutisch wirksamen Substanzen und/oder nicht-toxischen, inerten, therapeutisch verträglichen Trägermaterialien enthalten.

10 Die vorliegende Erfindung betrifft schliesslich die Verwendung solcher TNF-BP einerseits zur Herstellung pharmazeutischer Präparate bzw. andererseits zur Behandlung von Krankheiten, bevorzugt solchen, in deren Verlauf TNF involviert ist.

15 Ausgangsmaterial für die erfindungsgemässen TNF-BP sind ganz allgemein Zellen, die solche TNF-BP in membrangebundener Form enthalten und die dem Fachmann ohne Beschränkungen allgemein zugänglich sind, wie beispielsweise HL60- [ATCC Nr. CCL 240], U 937- [ATCC Nr. CRL 1593], SW 480- [ATCC Nr. CCL 228] und HEP2-Zellen [ATCC Nr. CCL 23]. Diese Zellen können nach bekannten Methoden des Standes der Technik [40] oder zum Erzielen hoher Zelldichten nach dem bereits allgemein und im Detail für HL60-Zellen in Beispiel 2 beschriebenen Verfahren kultiviert werden. TNF-BP können dann nach bekannten Methoden des Standes der Technik mittels geeigneter Detergenzien, beispielsweise Triton X-114, 1-O-n-Octyl- β -D-glucopyranosid (Octylglucosid), oder 3-[(3-Cholylamidopropyl)-dimethylammonio]-1-propan sulfonat (CHAPS), im besonderen mittels Triton X-100, aus den aus dem Medium abzentrifugierten und gewaschenen Zellen extrahiert werden. Zum Nachweis solcher TNF-BP können die üblicherweise verwendeten Nachweismethoden für TNF-BP, beispielsweise eine Polyäthylenglykol-induzierte Fällung des ¹²⁵I-TNF/TNF-BP-Komplexes [27], im besonderen Filterbindungstests mit radio-

aktiv markiertem TNF gemäss Beispiel 1. verwendet werden. Zur Gewinnung der erfindungsgemässen TNF-BP können die generell zur Reinigung von Proteinen, insbesondere von Membranproteinen, verwendeten Methoden des Standes der Technik, wie beispielsweise Ionenaustausch-Chromatographie, Gelfiltration, Affinitätschromatographie, HPLC und SDS-PAGE verwendet werden. Besonders bevorzugte Methoden zur Herstellung erfindungsgemässer TNF-BP sind Affinitätschromatographie, insbesondere mit TNF- α als an die Festphase gebundenen Liganden und Immunaффinitätschromatographie, HPLC und SDS-PAGE. Die Elution von mittels SDS-PAGE aufgetrennten TNF-BP Banden kann nach bekannten Methoden der Proteinchemie erfolgen, beispielsweise mittels Elektroelution nach Hunkapiller et al. [34], wobei nach heutigem Stand des Wissens die dort angegebenen Elektro-Dialysezeiten generell zu verdoppeln sind. Danach noch verbleibende Spuren von SDS können dann gemäss Bosserhoff et al. [50] entfernt werden.

Die so gereinigten TNF-BP können mittels der im Stand der Technik bekannten Methoden der Peptidchemie, wie beispielsweise N-terminale Aminosäuresequenzierung oder enzymatische wie chemische Peptidspaltung charakterisiert werden. Durch enzymatische oder chemische Spaltung erhaltene Fragmente können nach gängigen Methoden, wie beispielsweise HPLC, aufgetrennt und selbst wieder N-terminal sequenziert werden. Solche Fragmente, die selbst noch TNF binden, können mittels der obengenannten Nachweismethoden für TNF-BP identifiziert werden und sind ebenfalls Gegenstand der vorliegenden Erfindung.

Ausgehend von der so erhältlichen Aminosäuresequenzinformation oder den in Figur 1 dargestellten DNA- wie Aminosäuresequenzen können unter Beachtung der Degeneration des genetischen Codes nach im Stand der Technik bekannten Methoden geeignete Oligonukleotide hergestellt werden [51]. Mittels dieser können dann wiederum nach bekannten Methoden der Molekularbiologie [42,43] cDNA- oder genomische

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DNA-Banken nach Klonen, die für TNF-BP kodierende Nukleinsäuresequenzen enthalten, abgesucht werden. Ausserdem können mittels der Polymerase-Kettenreaktion (PCR) [49] cDNA-Fragmente kloniert werden, indem von zwei auseinanderliegenden, relativ kurzen Abschnitten der Aminosäuresequenz unter Beachtung des genetischen Codes vollständig degenerierte und in ihrer Komplementarität geeignete Oligonucleotide als "Primer" eingesetzt werden, wodurch das zwischen diesen beiden Sequenzen liegende Fragment amplifiziert und identifiziert werden kann. Die Bestimmung der Nukleotidsequenz eines derartigen Fragmentes ermöglicht eine unabhängige Bestimmung der Aminosäure-Sequenz des Proteinfragments, für das es kodiert. Die mittels der PCR erhältlichen cDNA-Fragmente können ebenfalls, wie bereits für die Oligonukleotide selbst beschrieben, nach bekannten Methoden zum Aufsuchen von für TNF-BP kodierende Nukleinsäuresequenzen enthaltenden Klonen aus cDNA- bzw. genomische DNA-Banken verwendet werden. Solche Nukleinsäuresequenzen können dann nach bekannten Methoden sequenziert werden [42]. Aufgrund der so bestimmten wie der für bestimmte Rezeptoren bereits bekannten Sequenzen, können solche Teilsequenzen, die für lösliche TNF-BP-Fragmente kodieren, bestimmt und mittels bekannter Methoden aus der Gesamtsequenz herausgeschnitten werden [42].

Die gesamte Sequenz oder solche Teilsequenzen können dann mittels bekannter Methoden in im Stand der Technik beschriebene Vektoren zu deren Vervielfältigung wie Expression in Prokaryoten integriert werden [42]. Geeignete prokaryotische Wirtsorganismen stellen beispielsweise gram-negative wie gram-positive Bakterien, wie beispielsweise E. coli Stämme, wie E. coli HB 101 [ATCC Nr. 33 694] oder E. coli W3110 [ATCC Nr. 27 325] oder B. subtilis Stämme dar.

Weiterhin können erfindungsgemässe Nukleinsäuresequenzen, die für TNF-BP sowie für TNF-BP-Fragmente kodieren, in geeignete Vektoren zur Vermehrung wie Expression in eukaryotischen Wirtszellen, wie beispielsweise Hefe,

Insekten- und Säugerzellen, mittels bekannter Methoden integriert werden. Expression solcher Sequenzen erfolgt bevorzugt in Säuger- wie Insektenzellen.

- 5 Ein typischer Expressionsvektor für Säugerzellen enthält ein effizientes Promotorelement, um eine gute Transkriptionsrate zu erzielen, die zu exprimierende DNA-Sequenz und Signale für eine effiziente Termination und Polyadenylierung des Transkripts. Weitere Elemente, die verwendet werden
10 können, sind "Enhancer", welche zu nochmals verstärkter Transkription führen und Sequenzen, welche z.B. eine längere Halbwertszeit der mRNA bewirken können.

- Die meisten Vektoren, die für eine transiente Expression
15 einer bestimmten DNA-Sequenz in Säugerzellen verwendet werden, enthalten den Replikationsursprung des SV40 Virus. In Zellen, die das T-Antigen des Virus exprimieren, (z.B. COS-Zellen), werden diese Vektoren stark vermehrt. Eine vorübergehende Expression ist aber nicht auf COS-Zellen
20 beschränkt. Im Prinzip kann jede transfektierbare Säugerzelllinie hierfür verwendet werden. Signale, die eine starke Transkription bewirken können, sind z.B. die frühen und späten Promotoren von SV40, der Promoter und Enhancer des "major immediate-early" Gens des HCMV (humaner Cytomegalovirus), die LTRs ("long terminal repeats") von Retroviren,
25 wie beispielsweise RSV, HIV und MMTV. Es können aber auch Signale von zellulären Genen, wie z.B. die Promotoren des Aktin- und Collagenase-Gens, verwendet werden.

- 30 Alternativ können aber auch stabile Zelllinien, die die spezifische DNA-Sequenz im Genom (Chromosom) integriert haben, erhalten werden. Hierzu wird die DNA-Sequenz zusammen mit einem selektierbaren Marker, z.B. Neomycin, Hygromycin, Dihydrofolat-Reduktase (dhfr) oder Hypoxanthin-Guanin-Phosphoribosyltransferase (hgpt) kotransfiziert. Die stabil ins
35 Chromosom eingebaute DNA-Sequenz kann auch noch stark vermehrt werden. Ein geeigneter Selektionsmarker hierfür ist

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beispielsweise die Dihydrofolat-Reduktase (dhfr). Säugerzellen (z.B. CHO-Zellen), welche kein intaktes dhfr-Gen enthalten, werden hierbei nach erfolgter Transfektion mit steigenden Mengen von Methotrexat inkubiert. Auf diese Weise
5 können Zelllinien erhalten werden, welche mehr als tausend Kopien der gewünschten DNA-Sequenz enthalten.

Säugerzellen, welche für die Expression verwendet werden können, sind z.B. Zellen der menschlichen Zelllinien Hela
10 [ATCC CCL2] und 293 [ATCC CRL 1573], sowie 3T3- [ATCC CCL 163] und L-Zellen, z.B. [ATCC CCL 149], (CHO)-Zellen [ATCC CCL 61], BHK [ATCC CCL 10]-Zellen sowie die CV 1 [ATCC CCL 70]- und die COS-Zelllinien [ATCC CRL 1650, CRL 1651].

15 Geeignete Expressionsvektoren umfassen beispielsweise Vektoren wie pBC12MI [ATCC 67 109], pSV2dhfr [ATCC 37 146], pSVL [Pharmacia, Uppsala, Sweden], pRSVcat [ATCC 37 152] und pMSG [Pharmacia, Uppsala, Sweden]. Besonders bevorzugte Vektoren sind die in Beispiel 9 verwendeten Vektoren "pK19"
20 und "pN123". Diese können aus den mit ihnen transformierten E. coli-Stämmen HB101(pK19) und HB101(pN123) nach bekannten Methoden isoliert werden [42]. Diese E. coli-Stämme wurden am 26. Januar 1990 bei der Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, BRD
25 unter DSM 5761 für HB101(pK19) und DMS 5764 für HB101(pN123) hinterlegt.

Die Art und Weise wie die Zellen transfektiert werden hängt vom gewählten Expressions- und Vektorsystem ab. Eine
30 Uebersicht über diese Methoden findet man z.B. bei Pollard et al., "DNA Transformation of Mammalian Cells" in "Methods in Molecular Biology" [Nucleic Acids Vol. 2, 1984, Walker, J.M., ed, Humana, Clifton, New Jersey]. Weitere Methoden findet man bei Chen und Okayama ["High-Efficiency Transfor-
35 mation of Mammalian Cells by Plasmid DNA", Molecular and Cell Biology 7, 2745-2752, 1987] und bei Felgner [Felgner et al., "Lipofectin: A highly efficient, lipid-mediated

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DNA-transfection procedure". Proc. Nat. Acad. Sci. USA 84, 7413-7417, 1987].

Zur Expression in Insektenzellen kann das Baculovirus-
5 -Expressions-System, welches schon für die Expression einer
Reihe von Proteinen erfolgreich eingesetzt worden ist (für
eine Uebersicht siehe Luckow and Summers, Bio/Technology 6,
47-55, 1988), verwendet werden. Rekombinante Proteine können
authentisch oder als Fusionsproteine hergestellt werden. Die
10 so hergestellten Proteine können auch modifiziert, wie
beispielsweise glykosyliert (Smith et al., Proc. Nat. Acad.
Sci. USA 82, 8404-8408, 1987) sein. Für die Herstellung
eines rekombinanten Baculovirus, der das gewünschte Protein
exprimiert, verwendet man einen sogenannten "Transfer-
15 vektor". Hierunter versteht man ein Plasmid, welches die
heterologe DNA-Sequenz unter der Kontrolle eines starken
Promoters, z.B. dem des Polyhedringens, enthält; wobei diese
auf beiden Seiten von viralen Sequenzen umgeben ist. Beson-
ders bevorzugte Vektoren sind die in Beispiel 10 verwendeten
20 Vektoren "pN113", "pN119" und "pN124". Diese können aus den
mit ihnen transformierten E. coli-Stämmen HB101(pN113),
HB101(pN119) und HB101(pN124) nach bekannten Methoden iso-
liert werden [42]. Diese E. coli-Stämme wurden am 26. Januar
1990 bei der Deutschen Sammlung von Mikroorganismen und
25 Zellkulturen GmbH (DSMZ) in Braunschweig, BRD, unter DSM 5762
für HB101(pN113), DSM 5763 für HB101(pN119) und DSM 5765 für
HB101(pN124) hinterlegt. Der Transfervektor wird dann
zusammen mit DNA des Wildtyp-Baculovirus in die Insekten-
zellen transfektiert. Die in den Zellen durch homologe
30 Rekombination entstehenden rekombinanten Viren können dann
nach bekannten Methoden identifiziert und isoliert werden.
Eine Uebersicht über das Baculovirus-Expressionssystem und
der dabei verwendeten Methoden findet man bei Luckow und
Summers [52]. -

35

Exprimierte TNF-BP wie ihre nichtlöslichen oder lös-
lichen Fragmente können dann nach im Stand der Technik

bekannten Methoden der Proteinchemie, wie beispielsweise den bereits auf Seiten 5-6 beschriebenen Verfahren, aus der Zellmasse oder den Kulturüberständen gereinigt werden.

5 Die erfindungsgemäss erhaltenen TNF-BP können auch als Antigene zur Erzeugung von poly- und monoklonalen Antikörpern nach bekannten Methoden der Technik [44,45] oder gemäss dem in Beispiel 3 beschriebenen Verfahren verwendet werden. Solche Antikörper, insbesondere monoklonale Antikörper gegen die 75 kD-TNF-BP-Spezies, sind ebenfalls Gegenstand der vorliegenden Erfindung. Solche gegen die 75 kD TNF-BP gerichtete Antikörper können durch dem Fachmann geläufige Modifikationen des in den Beispielen 4-6 im Detail beschriebenen Reinigungsverfahrens zur Isolierung von TNF-BP
15 eingesetzt werden.

Auf Grund der hohen Bindungsaffinität erfindungsgemässer TNF-BP für TNF (K_d -Werte in den Grössenordnungen von 10^{-9} - 10^{-10} M) können diese oder Fragmente davon als
20 Diagnostika zum Nachweis von TNF in Serum oder anderen Körperflüssigkeiten nach im Stand der Technik bekannten Methoden, beispielsweise in Festphasenbindungstests oder in Verbindung mit Anti-TNF-BP-Antikörpern in sogenannten "Sandwich"-Tests, eingesetzt werden.

25 Im übrigen können erfindungsgemässe TNF-BP einerseits zur Reinigung von TNF und andererseits zum Auffinden von TNF-Agonisten sowie TNF-Antagonisten nach im Stand der Technik bekannten Verfahren verwendet werden.

30 Die erfindungsgemässen TNF-BP sowie deren physiologisch verträgliche Salze, die nach im Stand der Technik bekannten Methoden hergestellt werden können, können auch zur Herstellung von pharmazeutischen Präparaten, vor allem solchen zur
35 Behandlung von Krankheiten, bei deren Verlauf TNF involviert ist, verwendet werden. Dazu kann eine oder mehrere der genannten Verbindungen, falls wünschenswert bzw. erforder-

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lich in Verbindung mit anderen pharmazeutisch aktiven Substanzen, mit den üblicherweise verwendeten festen oder flüssigen Trägermaterialien in bekannter Weise verarbeitet werden. Die Dosierung solcher Präparate kann unter Berücksichtigung der üblichen Kriterien in Analogie zu bereits verwendeten Präparaten ähnlicher Aktivität und Struktur erfolgen.

Nachdem die Erfindung vorstehend allgemein beschrieben worden ist, sollen die folgenden Beispiele Einzelheiten der Erfindung veranschaulichen, ohne dass diese dadurch in irgendeiner Weise eingeschränkt wird.

Beispiel 1

15

Nachweis von TNF-bindenden Proteinen

Die TNF-BP wurden in einem Filtertest mit humanem radiojodiertem ^{125}I -TNF nachgewiesen. TNF (46,47) wurde mit Na^{125}I (IMS40, Amersham, Amersham, England) und Iodo-Gen (#28600, Pierce Eurochemie, Oud-Beijerland, Niederlande) nach Praker und Speck [48] radioaktiv markiert. Zum Nachweis der TNF-BP wurden isolierte Membranen der Zellen oder ihre solubilisierten, angereicherten und gereinigten Fraktionen auf angefeuchtete Nitrocellulose-Filter (0.45 μ , BioRad, Richmond, California, USA) aufgetragen. Die Filter wurden dann in Pufferlösung mit 1% entfettetem Milchpulver blockiert und anschliessend mit $5 \cdot 10^5$ cpm/ml ^{125}I -TNF α (0.3 - $1.0 \cdot 10^8$ cpm/ μg) in zwei Ansätzen mit und ohne Beigabe von 5 $\mu\text{g}/\text{ml}$ nicht-markiertem TNF α inkubiert, gewaschen und luftgetrocknet. Die gebundene Radioaktivität wurde autoradiographisch semiquantitativ nachgewiesen oder in einem γ -Counter gezählt. Die spezifische ^{125}I -TNF- α -Bindung wurde nach Korrektur für unspezifische Bindung in Anwesenheit von unmarkiertem TNF- α im Ueberschuss ermittelt. Die spezifische TNF-Bindung im Filtertest wurde bei verschiedenen

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TNF-Konzentrationen gemessen und nach Scatchard analysiert [33], wobei ein K_d -Wert von $\sim 10^{-9}$ - 10^{-10} M ermittelt wurde.

5

Beispiel 2

Zellextrakte von HL-60-Zellen

10 HL60 Zellen [ATCC-Nr. CCL 240] wurden in experimentellem Labormassstab in einem RPMI 1640-Medium [GIBCO-Katalog Nr. 074-01800], das noch 2 g/l NaHCO_3 und 5% fötales Kälberserum enthielt, in einer 5% CO_2 -Atmosphäre kultiviert und anschliessend zentrifugiert.

15 Zum Erzielen hoher Zelldichten in technischem Massstab wurde folgendermassen verfahren. Die Züchtung wurde in einem 75 l Airliftfermenter (Fa. Chemap, Schweiz) mit 58 l Arbeitsvolumen durchgeführt. Hierfür wurde das Kassettenmembransystem "PROSTAK" (Millipore, Schweiz) mit einer
20 Membranfläche von $0,32 \text{ m}^2$ (1 Kassette) in den äusseren Zirkulationskreislauf integriert. Das Kulturmedium (siehe Tabelle 1) wurde mit einer Watson-Marlow Pumpe, Typ 603U, mit 5 l/min. umgepumpt. Nach einer Dampfsterilisation der Anlage, wobei das "PROSTAK" System im Autoklaven separat
25 sterilisiert wurde, wurde die Fermentation mit wachsenden HL-60 Zellen aus einem 20 l Airliftfermenter (Chemap) gestartet. Die Zellzüchtung im Impffermenter erfolgte im konventionellen Batchverfahren in dem Medium gemäss Tabelle 1 und einem Startzelltiter von 2×10^5 Zellen/ml. Nach 4
30 Tagen wurde der HL60 Ansatz mit einem Titer von $4,9 \times 10^6$ Zellen/ml in den 75 l Fermenter überführt. Der pH-Wert wurde bei 7.1 und der pO_2 Wert bei 25% Sättigung gehalten, wobei der Sauerstoffeintrag durch eine mikroporöse Fritte erfolgte. Nach anfänglicher Batchfermentation wurde am 2.
35 Tag die Perfusion bei einem Zelltiter von 4×10^6 Zellen/ml mit 30 l Mediumsaustausch pro Tag gestartet. Auf der Filtratseite der Membran wurde das konditionierte Medium

- abgezogen und durch den Zulauf von frischem Medium ersetzt. Das Zulaufmedium wurde wie folgt verstärkt: Primatone von 0,25% auf 0,35%, Glutamin von 5 mM auf 6 mM und Glucose von 4 g/l auf 6 g/l. Die Perfusionsrate wurde dann am 3. und 4. Tag auf 72 l Medium/Tag und am 5. Tag auf 100 l Medium/Tag erhöht. Nach 120 Stunden der kontinuierlichen Züchtung wurde die Fermentation beendet. Unter den gegebenen Fermentationsbedingungen erfolgte exponentielles Zellwachstum bis 40×10^6 Zellen/ml. Die Verdopplungszeit der Zellpopulation betrug bis 10×10^6 Zellen/ml 20-22 Stunden und stieg dann mit zunehmender Zelldichte auf 30-36 Stunden an. Der Anteil der lebenden Zellen lag während der gesamten Fermentationszeit bei 90-95%. Der HL-60 Ansatz wurde dann im Fermenter auf ca. 12°C heruntergekühlt und die Zellen durch Zentrifugation (Beckman-Zentrifuge [Modell J-6B, Rotor JS], 3000 rpm, 10 min., 4°C) geerntet.

Tabelle 1

20 HL-60 Medium

Komponenten	Konzentrationen mg/l
CaCl_2 (wasserfrei)	112,644
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	20
25 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	$0,498 \cdot 10^{-3}$
$\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$	0,02
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0,1668
KCl	336,72
KNO_3	0,0309
30 MgCl_2 (wasserfrei)	11,444
MgSO_4 (wasserfrei)	68,37
NaCl	5801,8
Na_2HPO_4 (wasserfrei)	188,408
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	75
35 $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$	$9,6 \cdot 10^{-3}$
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0,1726

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	D-Glucose	4000
	Glutathion (red.)	0.2
	Hepes-Puffer	2383,2
	Hypoxanthin	0,954
5	Linolsäure	0,0168
	Liponsäure	0,042
	Phenolrot	10,24
	Putrescin 2HCl	0,0322
	Na-Pyruvat	88
10	Thymidin	0,146
	Biotin	0,04666
	D-Ca-Pantothenat	2,546
	Cholinchlorid	5,792
	Folsäure	2,86
15	i-Inositol	11,32
	Niacinamid	2,6
	Nicotinamid	0,0074
	para-Aminobenzoessäure	0,2
	Pyridoxal HCl	2,4124
20	Pyridoxin HCl	0,2
	Riboflavin	0,2876
	Thiamin HCl	2,668
	Vitamin B ₁₂	0,2782
25	L-Alanin	11,78
	L-Asparaginsäure	10
	L-Asparagin H ₂ O	14,362
	L-Arginin	40
	L-Arginin HCl	92,6
30	L-Aspartat	33,32
	L-Cystin 2HCl	62,04
	L-Cystein HCl·H ₂ O	7,024
	L-Glutaminsäure	36,94
	L-Glutamin -	730
35	L-Glycin	21,5
	L-Histidin	3
	L-Histidin HCl·H ₂ O	27,392

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	L-Hydroxyprolin	4
	L-Isoleucin	73,788
	L-Leucin	75,62
	L-Lysin HCl	102,9
5	L-Methionin	21,896
	L-Phenylalanin	43,592
	L-Prolin	26,9
	L-Serin	31,3
	L-Threonin	53
10	L-Tryptophan	11,008
	L-Tyrosin•2Na	69,76
	L-Valin	62,74
	Penicillin/Streptomycin	100 U/ml
15	Insulin (human)	5 µg/ml
	Transferrin (human)	15 µg/ml
	Rinderserumalbumin	67 µg/ml
	Primatone RL (Sheffield Products, Norwich NY, USA)	0,25%
20	Pluronic F68 (Serva, Heidelberg, BRD)	0,01%
	Fötales Kälberserum	0,3-3%

Das Zentrifugat wurde mit isotonem Phosphatpuffer (PBS;
 25 0,2 g/l KCl, 0,2 g/l KH_2PO_4 , 8,0 g/l NaCl, 2,16 g/l
 $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$), der mit 5% Dimethylformamid, 10 mM
 Benzamidin, 100 E/ml Aprotinin, 10 µM Leupeptin, 1 µM
 Pepstatin, 1 mM o-Phenanthrolin, 5 mM Jodacetamid, 1 mM
 Phenylmethylsulfonylfluorid versetzt war (im folgenden als
 30 PBS-M bezeichnet), gewaschen. Die gewaschenen Zellen wurden
 bei einer Dichte von $2,5 \cdot 10^8$ Zellen/ml in PBS-M mit
 Triton X-100 (Endkonzentration 1,0%) extrahiert. Der Zell-
 extrakt wurde durch Zentrifugation geklärt ($15'000 \times g$,
 1 Stunde; $100'000 \times g$, 1 Stunde).

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Beispiel 3

Herstellung von monoklonalen (TNF-BP)-Antikörpern

5 Ein gemäss Beispiel 2 erhaltener Zentrifugationsüber-
stand aus Kultivierung von HL60-Zellen im experimentellen
Labormasstab wurde im Verhältnis 1:10 mit PBS verdünnt. Der
verdünnte Ueberstand wurde bei 4°C auf eine Säule aufge-
tragen (Flussrate: 0,2 ml/min.), die 2 ml Affigel 10 ent-
10 hielt (Bio Rad Katalog Nr. 153-6099), an das 20 mg rekombi-
nantes humanes TNF- α [Pennica, D. et al. (1984) Nature
312, 724; Shirai, T. et al. (1985) Nature 313, 803; Wang,
A.M. et al. (1985) Science 228, 149] gemäss den Empfehlungen
des Herstellers gekoppelt worden war. Die Säule wurde bei
15 4°C und einer Durchflussrate von 1 ml/min zuerst mit 20 ml
PBS, das 0,1% Triton X 114 enthielt und danach mit 20 ml PBS
gewaschen. So angereichertes TNF-BP wurde bei 22°C und einer
Flussrate von 2 ml/min mit 4 ml 100 mM Glycin, pH 2,8, 0,1%
Decylmaltosid eluiert. Das Eluat wurde in einer Centricon 30
20 Einheit [Amicon] auf 10 μ l konzentriert.

10 μ l dieses Eluates wurden mit 20 μ l vollständigem
Freundschen Adjuvans zu einer Emulsion gemischt. Je 10 μ l
der Emulsion wurden gemäss dem von Holmdahl, R. et al.
25 [(1985), J. Immunol. Methods 83, 379] beschriebenen Ver-
fahren an den Tagen 0, 7 und 12 in eine hintere Fusspfote
einer narkotisierten Balb/c-Maus injiziert.

Am Tag 14 wurde die immunisierte Maus getötet, der
30 popliteale Lymphknoten herausgenommen, zerkleinert und in
Iscove's Medium (IMEM, GIBCO Katalog Nr. 074-2200), das
2 g/l NaHCO₃ enthielt, durch wiederholtes Pipettieren
suspendiert. Gemäss einem modifizierten Verfahren von De
St.Groth und Scheidegger [J. Immunol. Methods (1980), 35, 1]
35 wurden 5×10^7 Zellen des Lymphknotens mit 5×10^7 PAI Maus-
-Myelomazellen (J.W. Stocker et al., Research Disclosure,
217, Mai 1982, 155-157), die sich in logarithmischem

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Wachstum befanden, fusioniert. Die Zellen wurden gemischt, durch Zentrifugation gesammelt und durch leichtes Schütteln in 2 ml 50% (v/v) Polyethylenglycol in IMEM bei Raumtemperatur resuspendiert und durch langsame Zugabe von 10 ml IMEM während 10 Minuten vorsichtigen Schüttelns verdünnt. Die Zellen wurden durch Zentrifugation gesammelt und in 200 ml vollständigem Medium [IMEM + 20% fötales Kälberserum, Glutamin (2.0 mM), 2-Mercaptoethanol (100 μ M), 100 μ M Hypoxanthine, 0.4 μ M Aminopterin und 16 μ M Thymidine (HAT)] resuspendiert. Die Suspension wurde auf 10 Gewebekulturschalen, die jeweils 96 Vertiefungen enthielten, verteilt und ohne Wechsel des Mediums bei 37°C in einer Atmosphäre von 5% CO₂ und einer relativen Luftfeuchtigkeit von 98% 11 Tage lang inkubiert.

Die Antikörper zeichnen sich aus durch ihre inhibierende Wirkung auf die TNF-Bindung an HL60-Zellen oder durch ihre Bindung an Antigen im Filtertest gemäss Beispiel 1. Zum Nachweis der biologischen Aktivität von anti(TNF-BP)-Antikörpern wurde folgendermassen verfahren: 5×10^6 HL60 oder U937-Zellen wurden in vollständigem RPMI 1640 Medium zusammen mit affinitätsgereinigten monoklonalen anti-(TNF-BP)-Antikörpern oder Kontrollantikörpern (d.h. solchen, die nicht gegen TNF-BP gerichtet sind) in einem Konzentrationsbereich von 1 ng/ml bis 10 μ g/ml inkubiert. Nach einer Stunde Inkubation bei 37°C wurden die Zellen durch Zentrifugation gesammelt und mit 4.5 ml PBS bei 0°C gewaschen. Sie wurden in 1 ml vollständigem RPMI 1640 Medium (Beispiel 2), das zusätzlich 0.1% Natriumazid und ¹²⁵I-TNF α (10⁶ cpm/ml) mit oder ohne Beigabe von unmarkiertem TNF α (s.o.) enthielt, resuspendiert. Die spezifische Radioaktivität des ¹²⁵I-TNF α betrug 700 Ci/mmol. Die Zellen wurden 2 Stunden bei 4°C inkubiert, gesammelt und 4 mal mit 4.5 ml PBS, das 1% BSA und 0.001% Triton X 100 (Fluka) enthielt, bei 0°C gewaschen. Die an die Zellen gebundene Radioaktivität wurde in einem γ -Scintillationszähler gemessen. In einem vergleichbaren Experiment wurde

die zellgebundene Radioaktivität von Zellen, die nicht mit anti-(TNF-BP)-Antikörpern behandelt worden waren, bestimmt (ungefähr 10 000 cpm/5x10⁶ Zellen).

5

Beispiel 4

Affinitätschromatographie

- Für die weitere Reinigung wurden jeweils ein gemäss
- 10 Beispiel 3 erhaltener monoklonaler anti-(55 kD TNF-BP)-Antikörper (2,8 mg/ml Gel), TNF α (3,0 mg/ml Gel) und Rinder-serumalbumin (BSA, 8,5 mg/ml Gel) gemäss den Vorschriften des Herstellers kovalent an CNBr-aktivierte Sepharose 4B (Pharmacia, Uppsala, Schweden) gekoppelt. Der gemäss
- 15 Beispiel 2 erhaltene Zellextrakt wurde über die so hergestellten und in der folgenden Reihenfolge hintereinandergeschalteten Säulen geleitet: BSA-Sepharose-Vorsäule, Immunaффinitätssäule [Anti-(55 kD-TNF-BP)-Antikörper], TNF α -Ligand-Affinitätssäule. Nach vollständigem Auftrag wurden
- 20 die beiden letztgenannten Säulen abgetrennt und einzeln für sich mit je 100 ml der folgenden Pufferlösungen gewaschen: (1) PBS, 1,0% Triton X-100, 10 mM Benzamidin, 100 E/ml Aprotinin; (2) PBS, 0,1% Triton X-100, 0,5M NaCl, 10 mM ATP, 10 mM Benzamidin, 100 E/ml Aprotinin; und (3) PBS, 0,1%
- 25 Triton X-100, 10 mM Benzamidin, 100 E/ml Aprotinin. Sowohl die Immun- als auch die TNF α -Ligand-Affinitätssäule wurden dann mit 100 mM Glycin pH 2,5, 100 mM NaCl, 0,2% Decyl-maltoside, 10 mM Benzamidin, 100 E/ml Aprotinin jede für sich eluiert. Die im Filtertest gemäss Beispiel 1 aktiven
- 30 Fraktionen jeder Säule wurden danach jeweils vereint und mit 1M Tris pH 8,0 neutralisiert.

- Die so vereinten TNF-BP-aktiven Fraktionen der Immun-
- 35 -Affinitätschromatographie einerseits und der TNF α -Ligand-Affinitätschromatographie andererseits wurden zur weiteren Reinigung nochmals auf je eine kleine TNF α -Ligand-Affinitätssäule aufgetragen. Danach wurden diese beiden Säulen mit

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je 40 ml von (1) PBS, 1.0% Triton X-100, 10 mM Benzamidin, 100 E/ml Aprotinin, (2) PBS, 0.1% Triton X-100, 0.5M NaCl, 10 mM ATP, 10mM Benzamidin, 100 E/ml Aprotinin, (3) PBS, 0.1% Triton X-100, (4) 50 mM Tris pH 7.5, 150 mM NaCl, 1.0% NP-40, 1.0% Desoxycholat, 0.1% SDS, (5) PBS, 0.2% Decylmaltosid gewaschen. Anschliessend wurden die Säulen mit 100 mM Glycin pH 2.5, 100 mM NaCl, 0.2% Decylmaltosid eluiert. Fraktionen von 0.5 ml von jeder Säule wurden für sich gesammelt und die gemäss Filtertest (Beispiel 1) aktiven Fraktionen von jeder Säule jeweils für sich vereint und in einer Centricon-Einheit (Amicon, Molekulargewichts-Ausschluss 10'000) aufkonzentriert.

Beispiel 5

15

Auftrennung mittels HPLC

Die gemäss Beispiel 4 erhaltenen aktiven Fraktionen wurden gemäss ihrer unterschiedlichen Herkunft (Immun- bzw. Ligand-Affinitätschromatographie) jeweils für sich auf C1/C8 Umkehrphasen-HPLC-Säulen (ProRPC, Pharmacia, 5x20 mm), die mit 0.1% Trifluoressigsäure, 0.1% Octylglucosid equilibriert worden waren, aufgetragen. Die Säulen wurden dann mit einem linearen Acetonitril-Gradienten (0-80%) im gleichen Puffer bei einem Fluss von 0.5 ml/min eluiert. Fraktionen von 1.0 ml wurden von jeder Säule gesammelt und die aktiven Fraktionen von jeder Säule für sich vereint (Nachweis gemäss Beispiel 1).

Beispiel 6

30

Auftrennung mittels SDS-PAGE

Die gemäss Beispiel 5 erhaltenen und gemäss Filtertest (Beispiel 1) aktiven Fraktionen wurden durch SDS-PAGE gemäss [34] weiter aufgetrennt. Dazu wurden die Proben in SDS-Probenpuffer während 3 Minuten auf 95°C erhitzt und anschliessend auf einem 12% Acrylamid-Trenngel mit einem

5 Stigen Sammelgel elektrophoretisch aufgetrennt. Als Referenz zur Bestimmung der scheinbaren Molekulargewichte auf dem SDS-PAGE Gel wurden die folgenden Eichproteine verwendet: Phosphorylase B (97,4 kD), BSA (66,2 kD), Ovalbumin (42,7 kD), Carboanhydrase (31,0 kD), Soya Trypsin-Inhibitor (21,5 kD) und Lysozym (14,4 kD).

10 Unter den genannten Bedingungen wurden für Proben, die gemäss Beispiel 4 durch TNF- α -Ligandenaffinitätschromatographie von Immunaflinitätschromatographieeluat erhalten und durch HPLC gemäss Beispiel 5 weiter aufgetrennt worden waren, zwei Banden von 55 kD und 51 kD sowie drei schwächere Banden von 38 kD, 36 kD und 34 kD erhalten. Diese Banden wurden in einem Mini Trans Blot System (BioRad, Richmond, 15 California, USA) elektrophoretisch während 1 Stunde bei 100 V in 25 mM Tris, 192 mM Glycin, 20% Methanol auf eine PVDF-Membran (Immobilon, Millipore, Bedford, Mass. USA) transferiert. Danach wurde die PVDF-Membran entweder mit 0,15% Serva-Blau (Serva, Heidelberg, BRD) in Methanol/Wasser/Eisessig (50/40/10 Volumenteile) auf Protein gefärbt oder mit entfettetem Milchpulver blockiert und anschliessend zum Nachweis von Banden mit TNF-BP-Aktivität mit ¹²⁵I-TNF α gemäss den in Beispiel 1 beschriebenen Filtertestbedingungen inkubiert. Dabei zeigte sich, dass alle in der 25 Proteinfärbung zur Darstellung gelangten Banden spezifisch TNF α banden. Alle diese Banden banden im Western Blot nach Towbin et al. [38] auch den gemäss Beispiel 3 hergestellten monoklonalen Anti-55kD-TNF-BP-Antikörper. Dabei wurde ein gemäss dem in Beispiel 1 beschriebenen Verfahren mit 30 Na¹²⁵I radioaktiv markierter, affinitätsgereinigter (Mausimmunglobulin-Sepharose-4B-Affinitätssäule) Kaninchen-anti-Maus-Immunglobulin-Antikörper zum autoradiographischen Nachweis dieses Antikörpers eingesetzt.

35 Proben, die gemäss Beispiel 4 durch zweimalige TNF- α -Ligandenaffinitätschromatographie des Durchlaufs der Immunaflinitätschromatographie erhalten und durch HPLC gemäss

Beispiel 5 weiter aufgetrennt worden waren, zeigten unter den oben spezifizierten SDS-PAGE- und Blottransfer-Bedingungen zwei zusätzliche Banden von 75 kD und 65 kD, die beide im Filtertest (Beispiel 1) spezifisch TNF banden. Im Western Blot gemäss Towbin et al. (s.o.) reagierten die Proteine dieser beiden Banden nicht mit dem gemäss Beispiel 3 hergestellten anti-(55 kD TNF-BP)-Antikörper. Sie reagierten allerdings mit einem monoklonalen Antikörper, der ausgehend von der 75 kD-Bande (anti-75 kD TNF-BP-Antikörper) gemäss Beispiel 3 erzeugt worden war.

Beispiel 7

Aminosäuresequenzanalyse

Zur Aminosäuresequenzanalyse wurden die gemäss Beispiel 5 erhaltenen und gemäss Filtertest (Beispiel 1) aktiven Fraktionen mittels der in Beispiel 6 beschriebenen, nun jedoch reduzierenden, SDS-PAGE Bedingungen (SDS-Probenpuffer mit 125 mM Dithiothreitol) aufgetrennt. Es wurden die gleichen Banden wie gemäss Beispiel 6 gefunden, die allerdings auf Grund der reduzierenden Bedingungen der SDS-PAGE im Vergleich zu Beispiel 6 alle um etwa 1-2 kD höhere Molekulargewichte zeigten. Diese Banden wurden dann gemäss Beispiel 6 auf PVDF-Membranen übertragen und mit 0,15% Serva-Blau in Methanol/Wasser/Eisessig (50/40/10 Volumenteile) während 1 Minute gefärbt, mit Methanol/Wasser/Eisessig (45/48/7 Volumenteile) entfärbt, mit Wasser gespült, luftgetrocknet und danach ausgeschnitten. Bei sämtlichen Schritten wurden zur Vermeidung von N-terminaler Blockierung die von Hunkapiller [34] angegebenen Bedingungen eingehalten. Zunächst wurden die gereinigten TNF-BP unverändert zur Aminosäuresequenzierung eingesetzt. Um zusätzliche Sequenzinformation zu erhalten, wurden die TNF-BP nach Reduktion und S-Carboxymethylierung [Jones, B.N. (1986) in "Methods of Protein Microcharacterisation", J.E. Shively, ed., Humana Press, Clifton NJ, 124-125] mit Bromcyan (Tarr,

G.E. in "Methods of Protein Microcharacterisation", 165-166, op.cit.), Trypsin und/oder Proteinase K gespalten und die Peptide mittels HPLC nach bekannten Methoden der Proteinchemie aufgetrennt. So vorbereitete Proben wurden dann in einem automatisierten Gasphasen-Mikrosequenzier-Gerät (Applied Biosystems Modell 470A, ABI, Foster City, Calif., USA) mit einem on-line nachgeschalteten automatisierten HPLC PTH-Aminosäureanalysator (Applied Biosystems Modell 120, ABI s.o.) sequenziert, wobei die folgenden Aminosäuresequenzen bestimmt wurden:

1., Für die 55 kD-Bande (gemäss nichtreduzierender SDS-PAGE):
Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile,
und
Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys
wobei X für einen Aminosäurerest steht, der nicht bestimmt werden konnte.

2., Für die 51 kD und die 38 kD-Banden (gemäss nichtreduzierender SDS-PAGE):
Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu

3., Für die 65 kD-Bande (gemäss nichtreduzierender SDS-PAGE):
Bei der N-terminalen Sequenzierung der 65 kD Bande wurden bis zum 15. Rest ohne Unterbrechung zwei parallele Sequenzen ermittelt. Da eine der beiden Sequenzen einer Teilsequenz des Ubiquitins [36,37] entsprach, wurde für die 65 kD-Bande die folgende Sequenz abgeleitet:

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Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys.

wobei X für einen Aminosäurerest steht, der nicht bestimmt werden konnte.

5

Weitere Peptidsequenzen für 75(65)kDa-TNP-BP wurden bestimmt:

Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu
10 und
Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu
und
Val-Phe-Cys-Thr
15 und
Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala
und
Leu-Cys-Ala-Pro
20 und
Val-Pro-His-Leu-Pro-Ala-Asp
und
Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro
25
wobei X für einen Aminosäurerest steht, der nicht bestimmt werden konnte.

Beispiel 8

30 Bestimmung von Basen-Sequenzen von komplementärer DNA (cDNA)

Ausgehend von der Aminosäuresequenz gemäss Formel IA wurden unter Berücksichtigung des genetischen Codes zu den Aminosäureresten 2-7 und 17-23 entsprechende, vollständig
35 degenerierte Oligonucleotide in geeigneter Komplementarität synthetisiert ("sense" and "antisense" Oligonucleotide).
Totale zelluläre RNA wurde aus HL60-Zellen isoliert [42,

43], und der erste cDNA-Strang durch Oligo-dT-Priming oder durch Priming mit dem "antisense" Oligonucleotid mittels eines cDNA-Synthese-Kits (RPN 1256, Amersham, Amersham, England) gemäss der Anleitung des Herstellers synthetisiert.

5 Dieser cDNA-Strang und die beiden synthetisierten degenerierten "sense" und "anti-sense" Oligonucleotide wurden in einer Polymerase-Kettenreaktion (PCR, Perkin Elmer Cetus, Norwalk, CT, USA gemäss Anleitung des Herstellers) dazu verwendet, die für die Aminosäure-Reste 8-16 (Formel 1A) codierende Basesequenz als cDNA-Fragment zu synthetisieren.

10 Die Basensequenz dieses cDNA-Fragmentes lautet:
 5'-AGGGAGAAGAGAGATAGTGTGTGTCCC-3'. Dieses cDNA-Fragment wurde als Probe verwendet, um nach bekannten Verfahren einen für das 55 kD TNF-BP codierenden cDNA-Klon in einer

15 λ gt11-cDNA-Genbank von menschlicher Placenta zu identifizieren (42,43). Dieser Klon wurde dann nach üblichen Methoden aus dem λ -Vektor geschnitten und in die Plasmide pUC18 (Pharmacia, Uppsala, Sweden) und pUC19 (Pharmacia, Uppsala, Sweden) und in die M13mpl8/M13mpl9 Bacteriophagen

20 (Pharmacia, Uppsala, Sweden) kloniert (42,43). Die Nukleotidsequenz dieses cDNA-Klons wurde mit einem Sequenase-Kit (U.S. Biochemical, Cleveland, Ohio, USA) nach den Angaben des Herstellers bestimmt. Die Nukleotidsequenz und die daraus abgeleitete Aminosäuresequenz für das 55 kD TNF-BP

25 und dessen Signalpeptid (Aminosäure "-28" bis Aminosäure "0") ist in Figur 1 mittels der im Stand der Technik üblichen Abkürzungen für Basen wie Aminosäuren dargestellt. Aus Sequenzvergleichen mit anderen, bereits bekannten Rezeptorproteinsequenzen lassen sich ungefähr 180 Aminosäuren enthaltende N-terminale wie 220 Aminosäure

30 enthaltende C-terminale Domänen, die von einer nach den Sequenzvergleichen typischen Transmembran-Region von 19 Aminosäuren (in Figur 1 unterstrichen) getrennt werden, bestimmen. Hypothetische Glykosylierungsstellen sind in

35 Figur 1 durch Sterne über der entsprechenden Aminosäure gekennzeichnet.

Beispiel 9Expression in COS 1-Zellen

- 5 Für die Expression in COS-Zellen wurden Vektoren ausgehend von dem Plasmid "pN11" konstruiert. Das Plasmid "pN11" enthält den effizienten Promotor und Enhancer des "major immediate-early" Gens des menschlichen Cytomegalovirus ("HCMV"; Boshart et al., Cell 41, 521-530, 1985).
- 10 Hinter dem Promotor befindet sich eine kurze DNA-Sequenz, welche mehrere Restriktionsschnittstellen enthält, die nur einmal im Plasmid vorkommen ("Polylinker"), u.a. die Schnittstellen für HindIII, BalI, BamHI und PvuII (siehe Sequenz).
- 15
- PvuII
- 5'-AAGCTTGGCCAGGATCCAGCTGACTGACTGATCGCGAGATC-3'
3'-TTCGAACCGGTCCTAGGTCGACTGACTGACTAGCGCTCTAG-5'
- 20 Hinter diesen Schnittstellen befinden sich drei Translations-Stopcodons in allen drei Leserastern. Hinter der Polylinkersequenz befindet sich das 2. Intron und das Polyadenylierungssignal des Präproinsulins der Ratte (Lomedico et al., Cell 18, 545-558, 1979). Das Plasmid enthält ferner den
- 25 Replikationsursprung des SV40 Virus sowie ein Fragment aus pBR322, das E. coli-Bakterien Ampicillin-Resistenz verleiht und die Replikation des Plasmids in E. coli ermöglicht.

- Zur Konstruktion des Expressionsvektors "pN123" wurde
- 30 dieses Plasmid "pN11" mit der Restriktionsendonuklease PvuII geschnitten und anschliessend mit alkalischer Phosphatase behandelt. Der dephosphorylierte Vektor wurde danach aus einem Agarosegel isoliert (V1). Die 5'-überhängenden Nukleotide des EcoRI-geschnittenen 1,3kb-Fragments der 55 kD
- 35 TNF-BP-cDNA (siehe Beispiel 8) wurden mit Hilfe von Klenow-Enzym aufgefüllt. Anschliessend wurde dieses Fragment aus einem Agarosegel isoliert (F1). Danach wurden V1 und F1

mittels T4-Ligase miteinander verbunden. E. coli HB101-Zellen wurden dann mit diesem Ligierungsansatz nach bekannten Methoden [42] transformiert. Mit Hilfe von Restriktionsanalysen und DNA-Sequenzierung nach bekannten Methoden [42] wurden Transformanten identifiziert, die mit einem Plasmid transformiert worden waren, welches das 1,3kb EcoRI-Fragment der 55 kD TNF-BP-cDNA in der für die Expression über den HCMV-Promotor korrekten Orientierung enthielt. Dieser Vektor erhielt die Bezeichnung "pN123".

Zur Konstruktion des Vektors "pK19" wurde folgendermassen verfahren. Ein DNA-Fragment, welches nur die für den extrazellulären Teil des 55 kD TNF-BP codierende cDNA enthält (Aminosäuren -28 bis 182 gemäss Figur 1) wurde mittels PCR-Technologie erhalten (Saiki et al., Science 230, 1350-1354, 1985, siehe auch Beispiel 8). Die folgenden Oligonukleotide wurden, um die für den extrazellulären Teil des 55 kD TNF-BP codierende cDNA aus "pN123" zu amplifizieren, verwendet:

BAMHI
5'-CACAGGGATCCATAGCTGTCTGGCATGGGCCTCTCCAC-3'

ASP718
3'-CGTGACTCCTGAGTCCGTGGTGTATTATCTCTAGACCATGGCCC-5'

Durch diese Oligonukleotide wurden ebenfalls zwei Stopkodons der Translation hinter Aminosäure 182 eingeführt. Das so amplifizierte DNA-Fragment wurde mit BamHI und Asp718 geschnitten, die hierbei entstandenen überstehenden Enden mit Hilfe des Klenow-Enzyms aufgefüllt und dieses Fragment anschliessend aus einem Agarosegel isoliert (F2). F2 wurde dann mit V1 ligiert und der gesamte Ansatz zur Transformation von E. coli HB101, wie bereits beschrieben, verwendet. Transformanten, die mit einem Plasmid transformiert worden waren, welches das DNA-Fragment in der für die Expression über den HCMV-Promotor korrekten Orientierung enthielten,

wurden mittels DNA-Sequenzierung (s.o.) identifiziert. Das daraus isolierte Plasmid erhielt die Bezeichnung "pK19".

Transfektion der COS-Zellen mit den Plasmiden "pN123" oder "pK19" wurde nach der von Pelgner et al. veröffentlichten Lipofections-Methode (Proc. Natl. Acad. Sci. USA 84, 7413-7417, 1987) durchgeführt. 72 Stunden nach erfolgter Transfektion wurden die mit "pN123" transfizierten Zellen nach bekannten Methoden mit 125 I-TNF α auf Bindung analysiert. Das Resultat der Scatchard-Analyse [Scatchard, G., Ann. N.Y. Acad. Sci. 51, 660, 1949] der so erhaltenen Bindungsdaten (Figur 2A) ist in Figur 2B dargestellt. Die Kulturüberstände der mit "pK19" transfizierten Zellen wurden in einem "Sandwich"-Test untersucht. Dazu wurden PVC-Microtiterplatten (Dynatech, Arlington, VA, USA) mit 100 μ l/Loch eines Kaninchen-anti-Maus Immunglobulins (10 μ g/ml PBS) sensibilisiert. Anschliessend wurde die Platte gewaschen und mit einem anti-55 kD TNF-BP-Antikörper, der gemäss Beispiel 3 durch seine Antigenbindung nachgewiesen und isoliert wurde, der aber die TNF-Bindung an Zellen nicht inhibiert, inkubiert (3 Stunden, 20°C). Die Platte wurde dann wieder gewaschen und über Nacht bei 4°C mit 100 μ l/Loch der Kulturüberstände (1:4 verdünnt mit 1% entfetteter Milchpulver enthaltendem Puffer A: 50 mM Tris/HCl pH 7.4, 140 mM NaCl, 5 mM EDTA, 0.02% Na-Azid) inkubiert. Die Platte wurde entleert und mit 125 I-TNF α enthaltendem Puffer A (10^6 cpm/ml, 100 μ l/Loch) mit oder ohne Zusatz von 2 μ g/ml unmarkiertem TNF während 2 Stunden bei 4°C inkubiert. Danach wurde die Platte 4 mal mit PBS gewaschen, die einzelnen Löcher wurden ausgeschnitten und in einem γ -Zähler gemessen. Die Resultate von 5 parallelen Transfektionen (Säulen # 2, 3, 4, 6 und 7), von zwei Kontroll-Transfektionen mit dem pN11-Vektor (Säulen # 1, 5) und von einer Kontrolle mit HL60-Zell-Lysat (Säule # 8) sind in Figur 3 dargestellt.

Beispiel 10

Expression in Insektenzellen

5 Für die Expression in einem Baculovirus-Expressions-
system wurde von dem Plasmid "pVL941" (Luckow und Summers,
1989, "High Level Expression of Nonfused Foreign Genes with
Autographa californica Nuclear Polyhedrosis virus Expression
10 Vectors", Virology 170, 31-39) ausgegangen und dieses
folgendermassen modifiziert. Es wurde die einzige EcoRI-
-Restriktionsschnittstelle in "pVL941" entfernt, indem das
Plasmid mit EcoRI geschnitten und die überstehenden 5'-Enden
mit Klenow-Enzym aufgefüllt wurden. Das hieraus erhaltene
Plasmid pVL941/E- wurde mit BamHI und Asp718 verdaut und der
15 Vektorrumpf anschliessend aus einem Agarosegel isoliert.
Dieses Fragment wurde mit einem synthetischen Oligonukleotid
der folgenden Sequenz ligiert:

	BamHI	EcoRI	Asp718	
20	5' -	GATCCAGAATTCATAATAG	-	3'
	3' -	GTCTTAAGTATTATCCATG	-	5'

E. coli HB101 wurde mit dem Ligierungsansatz trans-
formiert und Transformanten, die ein Plasmid enthielten, in
25 welches das Oligonukleotid korrekt eingebaut worden war,
wurden durch Restriktionsanalyse und DNA-Sequenzierung nach
bekannten Methoden (s.o.) identifiziert; dieses Plasmid
wurde "pNR704" genannt. Zur Konstruktion des Transfervektors
"pN113" wurde dieses Plasmid "pNR704" mit EcoRI geschnitten,
30 mit alkalischer Phosphatase behandelt und der so erzeugte
Vektorrumpf (V2) anschliessend aus einem Agarosegel iso-
liert. Das wie oben mit EcoRI geschnittene 1.3 kb-Fragment
der 55 kD TNF-BP-cDNA wurde mit Fragment V2 ligiert. Mit
diesem Ligierungsansatz erhaltene Transformanten, die ein
35 Plasmid enthielten, welches das cDNA-Insert in der korrekten
Orientierung für die Expression über den Polyhedrinpromotor
enthielten, wurden identifiziert (s.o.). Der daraus iso-

lierte Vektor erhielt die Bezeichnung "pN113".

Zur Konstruktion des Transfervektors "pN119" wurde folgendermassen vorgegangen. Das 1,3 kb EcoRI/EcoRI-Fragment
5 der 55 kD TNF-BP cDNA in dem "pUC19"-Plasmid (siehe Beispiel 8) wurde mit BanI verdaut und mit dem folgenden synthetischen Oligonukleotid ligiert:

	BanI	Asp718
10	5' - GCACCACATAATAGAGATCTGGTACCGGGAA - 3'	
	3' - GTGTATTATCTCTAGACCATGGCCC - 5'	

Mit dem obigen Adaptor werden zwei Stopcodons der Translation hinter Aminosäure 182 und eine Schnittstelle für
15 die Restriktionsendonuklease Asp718 eingebaut. Nach erfolgter Ligation wurde der Ansatz mit EcoRI und Asp718 verdaut und das partielle 55 kD TNF-BP-Fragment (F3) isoliert. Weiterhin wurde das ebenfalls mit Asp718 und EcoRI geschnittene Plasmid "pNR704" mit F3 ligiert und der Ligierungsansatz in E. coli HB101 transformiert. Die Identifikation der Transformanten, welche ein Plasmid enthielten, in
20 das die partielle 55 kD TNF-BP cDNA korrekt für die Expression integriert worden war, erfolgte wie bereits beschrieben. Das aus diesen Transformanten isolierte Plasmid erhielt
25 den Namen "pN119".

Zur Konstruktion des Transfervektors "pN124" wurde folgendermassen vorgegangen. Das in Beispiel 9 beschriebene, für den extrazellulären Teil des 55 kD TNF-BP codierende
30 cDNA-Fragment wurde mit den angegebenen Oligonukleotiden mit Hilfe der PCR-Technologie, wie in Beispiel 9 beschrieben, amplifiziert. Dieses Fragment wurde mit BamHI und Asp718 geschnitten und aus einem Agarosegel isoliert (F4). Das Plasmid "pNR704" wurde ebenfalls mit BamHI und Asp718
35 geschnitten und der Vektorrumpf (V4) wurde isoliert (s.o.). Die Fragmente V4 und F4 wurden ligiert, E. coli HB101 damit transformiert und der rekombinante Transfervektor "pN124"

wurde, wie beschrieben, identifiziert und isoliert.

Zur Transfektion der Insektenzellen wurde folgender-
massen vorgegangen. 3 µg des Transfervektors "pN113"
wurden mit 1 µg DNA des Autographa californica-Nuklear-
polyhedrosisvirus (AcMNPV) (EP 127839) in Sf9-Zellen (ATCC
CRL 1711) transfektiert. Polyhedrin negative Viren wurden
identifiziert und aus "Plaques" gereinigt [52]. Mit diesen
rekombinanten Viren wurden wiederum Sf9 Zellen wie in [52]
beschrieben, infiziert. Nach 3 Tagen in Kultur wurden die
infizierten Zellen auf Bindung von TNF mittels ^{125}I -TNF α
untersucht. Dazu wurden die transfektierten Zellen mit einer
Pasteurpipette von der Zellkulturschale abgewaschen und bei
einer Zelldichte von 5×10^6 Zellen/ml Kulturmedium [52],
das 10 ng/ml ^{125}I -TNF- α enthielt, sowohl in Anwesenheit
wie Abwesenheit von 5 µg/ml nichtmarkiertem TNF- α
resuspendiert und 2 Stunden auf Eis inkubiert. Danach wurden
die Zellen mit reinem Kulturmedium gewaschen und die zell-
gebundene Radioaktivität in einem γ -Zähler gezählt (siehe
Tabelle 2).

Tabelle 2

25	Zellen	Zellgebundene Radioaktivität pro 10^6 Zellen
	nichtinfizierte Zellen (Kontrolle)	60 cpm
	infizierte Zellen	1600 ± 330 cpm ¹⁾

30
1) Mittelwert und Standardabweichung aus 4 Experimenten

00-80-12 M

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Patentansprüche

1. Nichtlösliche Proteine und lösliche oder nicht-
lösliche Fragmente davon, die TNF binden, in homogener Form,
5 sowie deren physiologisch verträgliche Salze.

2. Verbindungen gemäss Anspruch 1, die durch Molekular-
gewichte gemäss SDS-PAGE unter nichtreduzierenden Be-
dingungen von etwa 55 kD und 75 kD charakterisiert sind.

10 3. Verbindungen gemäss einem der Ansprüche 1 und 2, die
wenigstens eine der folgenden Aminosäuresequenzen enthalten:

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-
15 Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile;

Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys;

Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-
20 Gly-Ser-Thr-Cys;

Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu;

Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-
25 Glu-Lys-Pro-Leu;

Val-Phe-Cys-Thr;

Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-
30 Glu-Ala;

Leu-Cys-Ala-Pro;

Val-Pro-His-Leu-Pro-Ala-Asp;
35

Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro

wobei X für einen nicht bestimmten Aminosäurerest steht.

5 4. Ein Verfahren zur Isolierung einer Verbindung gemäss
einem der Ansprüche 1-3, dadurch gekennzeichnet, dass man im
wesentlichen die folgenden Reinigungsschritte nacheinander
ausführt: Herstellung eines Zellextraktes, Immunaффinitäts-
chromatographie und/oder ein- oder mehrfache Liganden-
10 affinitätschromatographie, HPLC und präparative SDS-PAGE.

5. Pharmazeutische Präparate, dadurch gekennzeichnet,
dass sie eine oder mehrere Verbindung(en) gemäss einem der
Ansprüche 1-3, gewünschtenfalls in Kombination mit weiteren
15 pharmazeutisch wirksamen Substanzen und/oder nicht-toxischen,
inerten, therapeutisch verträglichen Trägermaterialien
enthalten.

6. Verwendung einer Verbindung gemäss einem der
20 Ansprüche 1-3 zur Herstellung pharmazeutischer Präparate
bzw. zur Behandlung von Krankheiten, bevorzugt solchen, bei
denen TNF involviert ist.

7. Gegen eine Verbindung gemäss Ansprüche 1-3 gerichtete
25 Antikörper.

8. DNA-Sequenzen, die für Proteine und lösliche oder
nichtlösliche Fragmente davon, die TNF binden, kodieren.

30 9. Von DNA-Sequenzen gemäss Anspruch 8 kodierte rekombinante Proteine.

10. Vektoren, die DNA-Sequenzen gemäss Anspruch 8
enthalten und zur Expression der von diesen DNA-Sequenzen
35 kodierten Proteinen in prokaryotischen- wie eukaryotischen
Wirtssystemen geeignet sind.

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Abc. 10

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11. Prokaryotische- wie eukaryotische Wirtssysteme, die mit einem Vektor gemäss Anspruch 10 transformiert worden sind.

5 12. Ein Verfahren zur Herstellung von Verbindungen gemäss Anspruch 9, das dadurch gekennzeichnet ist, dass man ein wie in Anspruch 11 beanspruchtes transformiertes Wirtssystem in einem geeigneten Medium kultiviert und aus dem Wirtssystem selbst oder dem Medium solche Verbindungen
10 isoliert.

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Unvariable Exemplar
Exemplaire Invariable
Esemplare Immutabile

Figur 1

43105900

-185 GAATTCGGGGGGTTCAAGATCACTGGGACCAGGCCGTGATCTCTATGCCCGAGTCTCAA
-125 CCCTCAACTGTCACCCCAAGGCACTTGGGACGTCCTGGACAGACCGAGTCCCGGGAAGCC
-65 CCAGCACTGCCGCTGCCCACTGCCCTGAGCCCAATGGGGGAGTGAGAGGCCATAGCTG
-28.
-30 MetGlyLeuSerThrValProAspLeuLeuLeuProLeuValLeuLeuGluLeu
-5 TCTGGCATGGGCCTCTCCACCGTGCCTGACCTGCTGCTGCCGCTGGTGCTCCTGGAGCTG
+1
-10 LeuValGlyIleTyrProSerGlyValIleGlyLeuValProHisLeuGlyAspArgGlu
55 TTGGTGGAATATACCCCTCAGGGGTTATTGGACTGGTCCCTCACCTAGGGGACAGGGAG

10 LysArgAspSerValCysProGlnGlyLysTyrIleHisProGlnAsnAsnSerIleCys
115 AAGAGAGATAGTGTGTGTCCCAAGGAAAATATATCCACCCTCAAAATAATTGATTTC
30 CysThrLysCysHisLysGlyThrTyrLeuTyrAsnAspCysProGlyProGlyGlnAsp
175 TGTACCAAGTGCCACAAGGAACCTACTGTACAATGACTGTCCAGGCCCGGGGCAGGAT
50 ThrAspCysArgGluCysGluSerGlySerPheThrAlaSerGluAsnHisLeuArgHis
235 ACGGACTGCAGGGAGTGTGAGAGCGGCTCCTTCACCGCTTCAGAAAACCACCTCAGACAC
70 CysLeuSerCysSerLysCysArgLysGluMetGlyGlnValGluIleSerSerCysThr
295 TGCCTCAGCTGCTCCAAATGCCGAAAGGAAATGGGTGAGGTGGAGATCTCTTCTGCACA
90 ValAspArgAspThrValCysGlyCysArgLysAsnGlnTyrArgHisTyrTrpSerGlu
355 GTGGACCGGGACACCGTGTGTGGCTGCAGGAAGAACCAGTACCGGCATTATTGGAGTGAA

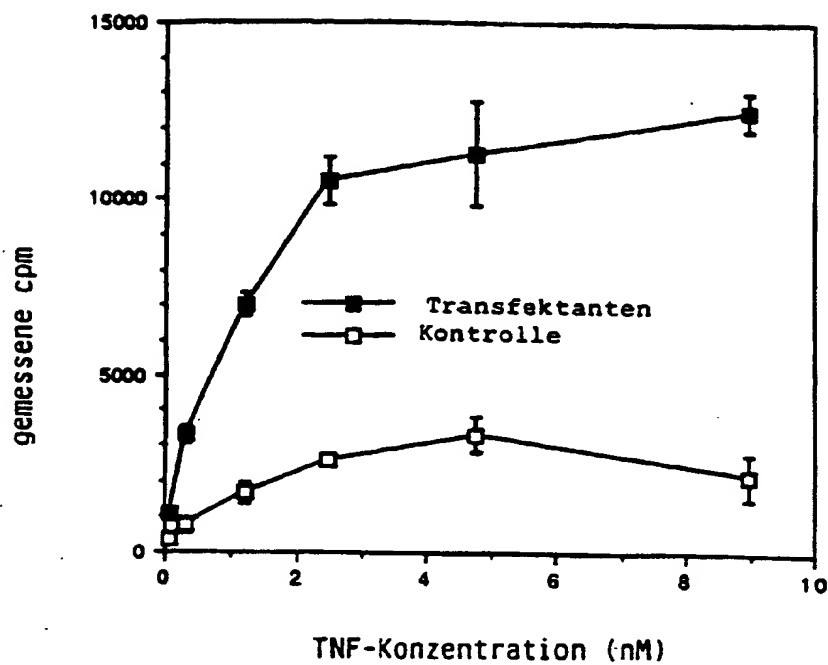
110 AsnLeuPheGlnCysPheAsnCysSerLeuCysLeuAsnGlyThrValHisLeuSerCys
415 AACCTTTTCCAGTGCTTCAATTGCAGCCTCTGCCTCAATGGGACCGTGCACCTCTCCTGC
130 GlnGluLysGlnAsnThrValCysThrCysHisAlaGlyPhePheLeuArgGluAsnGlu
475 CAGGAGAAACAGAACACCGTGTGCACCTGCCATGCAGGTTTCTTTCTAAGAGAAAACGAG
150 CysValSerCysSerAsnCysLysLysSerLeuGluCysThrLysLeuCysLeuProGln
535 TGTGTCTCCTGTAGTAAGTGTAGAAAAGCCTGGAGTGCACGAAGTTGTGCCTACCCAG
170 IleGluAsnValLysGlyThrGluAspSerGlyThrThrValLeuLeuProLeuValIle
595 ATTGAGAAATGTTAAGGGCACTGAGGACTCAGGCACCACAGTGCTGTTGCCCTGGTCATT
190 PhePheGlyLeuCysLeuLeuSerLeuLeuPheIleGlyLeuMetTyrArgTyrGlnArg
655 TTCTTTGGTCTTTGCCTTTTATCCTCCTCTTCATTGGTTTAAATGTATCGCTACCAACGG
210 TrpLysSerLysLeuTyrSerIleValCysGlyLysSerThrProGluLysGluGlyGlu
715 TGAAGTCCAAGCTCTACTCCATTGTTTGTGGGAAATCGACACCTGAAAAAGAGGGGAG

230 LeuGluGlyThrThrThrLysProLeuAlaProAsnProSerPheSerProThrProGly
775 CTTGAAGGAAGTACTACTAAGCCCTGGCCCCAAACCCAAGCTTCAGTCCCACTCCAGGC
250 PheThrProThrLeuGlyPheSerProValProSerSerThrPheThrSerSerSerThr
835 TTCACCCCAACCTGGGCTTCAGTCCCGTGCCAGTTCCACCTTCACCTCCAGCTCCACC
270 TyrThrProGlyAspCysProAsnPheAlaAlaProArgArgGluValAlaProProTyr
895 TATACCCCGGTGACTGTCCCAACTTTGCGGCTCCCCGAGAGAGGTGGCACCACCTAT
290 GlnGlyAlaAspProIleLeuAlaThrAlaLeuAlaSerAspProIleProAsnProLeu
955 GAGGGGCTGACCCCATCTTGCGACAGCCCTGCTTCCGACCCCATCCCCAACCCCTT

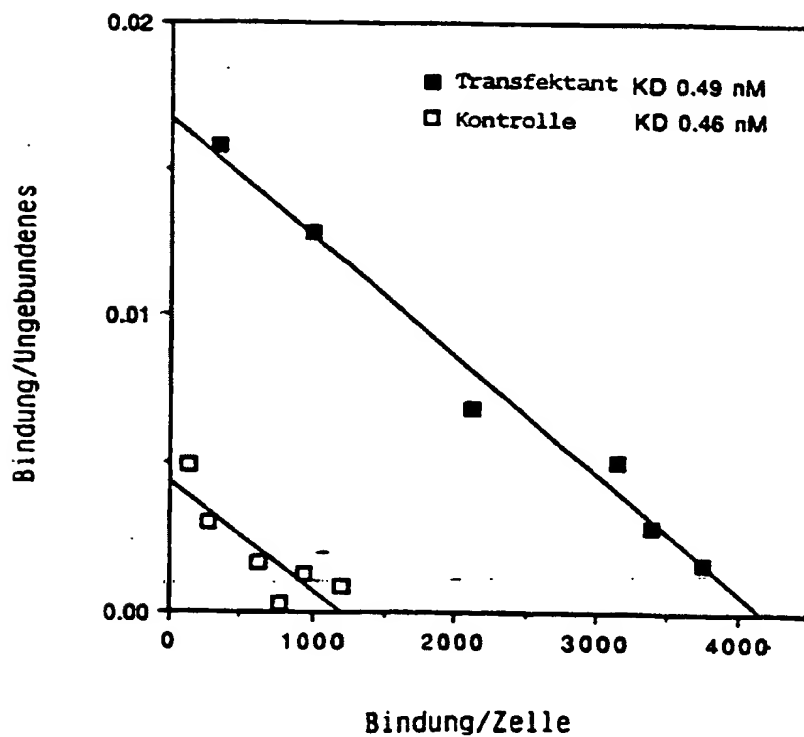
310 GlnLysTrpGluAspSerAlaHisLysProGlnSerLeuAspThrAspAspProAlaThr
1015 CAGAAGTGGGAGGACAGCGCCCAAGCCACAGAGCCTAGACACTGATGACCCCGCGACC
330 LeuTyrAlaValValGluAsnValProProLeuArgTrpLysGluPheValArgArgLeu
1075 CTGTACGCCGTGGTGGAGAACGTGCCCCGTTGCGCTGGAAGGAATTCGTGCGGCGCCTA
350 GlyLeuSerAspHisGluIleAspArgLeuGluLeuGlnAsnGlyArgCysLeuArgGlu
1135 GGGCTGAGCGACCACGAGATCGATCGGCTGGAGCTGCAGAACGGGCGCTGCCTGCGCGAG
370 AlaGlnTyrSerMetLeuAlaThrTrpArgArgArgThrProArgArgGluAlaThrLeu
1195 GCGCAATACAGCATGCTGGCGACCTGGAGGCGCGCACGCCGCGGCGGAGGCCACGCTG
390 GluLeuLeuGlyArgValLeuArgAspMetAspLeuLeuGlyCysLeuGluAspIleGlu
1255 GAGCTGCTGGGACGCGTGCTCCGCGACATGGACCTGCTGGGCTGCCTGGAGGACATCGAG
410 GluAlaLeuCysGlyProAlaAlaLeuProProAlaProSerLeuLeuArg
1315 GAGGCGCTTTGCGGCCCGCCGCCCTCCCGCCCGCGCCAGTCTTCTCAGATGAGGCTGC
1375 GCGGCTGCGGGCAGCTCTAAGGACCGTCTGCGAGATCGCCTTCCAACCCCACTTTTTTC
1435 TGGAAAGGAGGGGTCTGCGAGGGCAAGCAGGAGCTAGCAGCCGCCTACTTGGTGCTAAC
1495 CCCTCGATGTACATAGCTTTTCTCAGCTGCCTGCGCGCCGCGACAGTCAGCGCTGTGCG
1555 CGCGGAGAGAGGTGCGCCGTGGGCTCAAGAGCCTGAGTGGGTGGTTTGGGAGGATGAGGG
1615 ACGCTATGCCTCATGCCCCTTTTGGGTGTCTCACCAGCAAGGCTGCTCGGGGGCCCCCTG
1675 GTTCGTCCCTGAGCCTTTTTACAGTGCATAAGCAGTTTTTTTTGTGTTTTGTTTT
1735 GTTTTGTGTTTTAAATCAATCATGTTACACTAATAGAACTTGGCACTCCTGTGCCCTCTG
1795 CCTGGACAAGCACATAGCAAGCTGAACTGTCTAAGGCAGGGGCGAGCACGGAACAATGG
1855 GGCCTTCAGCTGGAGCTGTGGACTTTTGACATACACTAAAATTCTGAAGTTAAAAAAA
1915 AACCCGAATTC

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Figur 2A

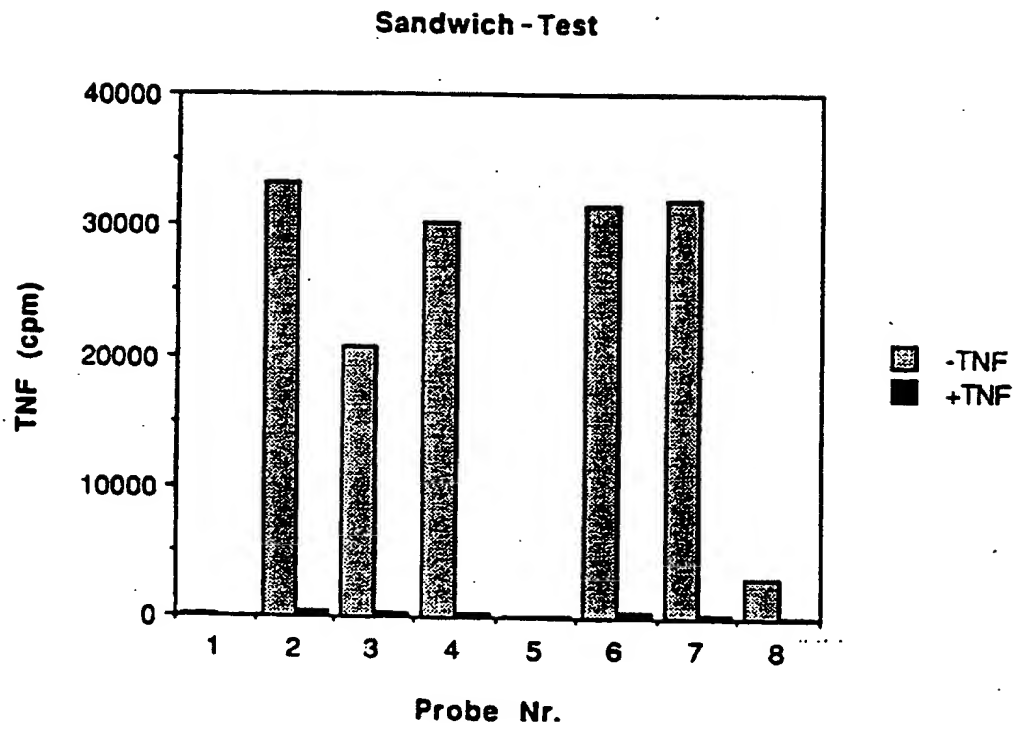


Figur 2B



100-443887-100

Figur 3





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SCHWEIZERISCHE EIDGENOSSENSCHAFT CONFÉDÉRATION SUISSE CONFEDERAZIONE SVIZZERA

Bescheinigung

Die beiliegenden Akten stimmen überein mit den ursprünglichen technischen Unterlagen des auf der nächsten Seite bezeichneten Patentgesuches für die Schweiz und Liechtenstein. *

Attestation

Les documents ci-joints sont conformes aux pièces techniques originales de la demande de brevet pour la Suisse et le Liechtenstein * spécifiée à la page suivante.

Attestazione

Gli uniti documenti sono conformi agli atti tecnici originali della domanda di brevetto per la Svizzera e il Liechtenstein * specificata nella pagina seguente.

Bern, 15. Juni 1990

Bundesamt für geistiges Eigentum
Office fédéral de la propriété intellectuelle
Ufficio federale della proprietà intellettuale

Der Sektionschef / Le chef de section / Il capo di sezione

Grünig

* Die Schweiz und das Fürstentum Liechtenstein bilden ein einheitliches Schutzgebiet. Der Schutz kann deshalb nur für beide Länder gemeinsam beantragt werden.

* La Suisse et le Principauté de Liechtenstein constituent un territoire unitaire de protection. La protection ne peut donc être revendiquée que pour l'ensemble des deux États.

* La Svizzera e il Principato di Liechtenstein formano un unico territorio di protezione. La protezione può dunque essere rivendicata solamente per l'insieme dei due Stati.

N 31.08.90

Voraussichtliche Klasse(n): C07K/A61K/C07H/C12N Patentgesuch Nr. 01 347/90-6 ✓

Patent-
bewerber: F. Hoffmann-La Roche AG
4002 Grenzacherstrasse 124
Basel
Postfach
Schweiz

Titel: TNF-bindende Proteine.

Datum der
Anmeldung: 20.04.90 ✓

Priorität: -

Referenz: RAN 4105/125-002

11.01.08.90

F. HOFFMANN-LA ROCHE AG, Basel/Schweiz

RAN 4105/125-002

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TNF-bindende Proteine

15 Tumor Nekrosis Faktor α (TNF α , auch Cachectin), auf
Grund seiner haemorrhagisch-nekrotisierenden Wirkung auf
bestimmte Tumoren entdeckt, und Lymphotoxin (TNF β) sind zwei
nahe verwandte Peptidfaktoren [3] aus der Klasse der Lympho-
kine/Cytokine, die im folgenden beide als TNF bezeichnet
20 werden [siehe Uebersichtsarbeiten 2 und 3]. TNF verfügt über
ein breites zelluläres Wirkungsspektrum. Beispielsweise
besitzt TNF inhibierende oder cytotoxische Wirkung auf eine
Reihe von Tumorzelllinien [2,3], stimuliert die Prolifera-
tion von Fibroblasten und die phagozytierende/cytotoxische
25 Aktivität von myeloischen Zellen [4,5,6], induziert
Adhäsionsmoleküle in Endothelzellen oder übt eine inhibie-
rende Wirkung auf Endothel aus [7,8,9,10], inhibiert die
Synthese von spezifischen Enzymen in Adipozyten [11] und
induziert die Expression von Histokompatibilitätsantigenen
30 [12]. Manche dieser TNF-Wirkungen werden über eine Induktion
von anderen Faktoren oder durch synergistische Effekte mit
anderen Faktoren, wie beispielsweise Interferonen oder
Interleukinen erzielt [13-16].

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TNF ist bei einer Reihe von pathologischen Zuständen, beispielsweise Schockzuständen bei Meningococcen-Sepsis [17], bei der Entwicklung von Autoimmun-Glomerulonephritis bei Mäusen [18] oder bei cerebraler Malaria bei Mäusen [19] und beim Menschen [41] involviert. Ganz allgemein scheinen die toxischen Wirkungen von Endotoxin durch TNF vermittelt zu sein [20]. Weiterhin kann TNF wie Interleukin-1 Fieber auslösen [39]. Auf Grund der pleiotropen funktionellen Eigenschaften von TNF kann man annehmen, dass TNF in Wechselwirkung mit anderen Cytokinen bei einer ganzen Reihe weiterer pathologischer Zustände als Mediator von Immunantwort, Entzündung oder anderen Prozessen beteiligt ist.

Diese biologischen Effekte werden durch TNF über spezifische Rezeptoren vermittelt, wobei nach heutigem Wissensstand sowohl TNF α wie TNF β an die gleichen Rezeptoren binden [21]. Verschiedene Zelltypen unterscheiden sich in der Anzahl von TNF-Rezeptoren [22,23,24]. Solche ganz allgemein gesprochen TNF-bindenden Proteine (TNF-BP) wurden durch kovalente Bindung an radioaktiv markiertes TNF nachgewiesen [24-29], wobei die folgenden scheinbaren Molekulargewichte der erhaltenen TNF/TNF-BP-Komplexe ermittelt wurden: 95/100 kD und 75 kD [24], 95 kD und 75 kD [25], 138 kD, 90 kD, 75 kD und 54 kD [26], 100 \pm 5 kD [27], 97 kD und 70 kD [28] und 145 kD [29]. Mittels anti-TNF-Antikörper-Immunoaffinitätschromatographie und präparativer SDS-Polyacrylamidgelelektrophorese (SDS-PAGE) konnte ein solcher TNF/TNF-BP-Komplex isoliert werden [27]. Die reduktive Spaltung dieses Komplexes und anschliessende SDS-PAGE-Analyse ergab mehrere Banden, die allerdings nicht auf TNF-Bindeaktivität getestet wurden. Da die spezifischen Bedingungen, die zu der Spaltung des Komplexes verwendet werden müssen, zur Inaktivierung des Bindeproteins führen [31], ist letzteres auch nicht möglich gewesen. Die Anreicherung von löslichen TNF-BP aus dem humanen Serum oder Urin mittels Ionenaustauscher-Chromatographie und Gelfiltration (Molekulargewichte im Bereich von 50 kD) wurde von

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Olsson et al. beschrieben [30].

5 Brockhaus et al. [32] erhielten durch TNF α -Liganden-
affinitätschromatographie und HPLC aus Membranextrakten von
HL60-Zellen eine angereicherte TNF-BP-Präparation, die
wiederum als Antigenpräparation zur Herstellung von mono-
klonalen Antikörpern gegen TNF-BP verwendet wurde. Unter
Verwendung eines solchen immobilisierten Antikörpers (Immun-
affinitätschromatographie) wurde mittels TNF α -Liganden-
10 affinitätschromatographie und HPLC von Loetscher und
Brockhaus [31] aus einem Extrakt von humaner Placenta eine
angereicherte Präparation von TNF-BP erhalten, die in der
SDS-PAGE-Analyse eine starke breite Bande bei 35 kD, eine
schwache Bande bei etwa 40 kD und eine sehr schwache Bande
15 im Bereich zwischen 55 kD und 60 kD ergab. Im übrigen zeigte
das Gel im Bereich von 33 kD bis 40 kD einen Protein-
hintergrundschmier. Die Bedeutung der so erhaltenen Protein-
banden war jedoch im Hinblick auf die Heterogenität des
verwendeten Ausgangsmaterials (Placenta-Gewebe; vereinigt
20 Material aus mehreren Placenten) nicht klar.

Gegenstand der vorliegenden Erfindung sind nichtlösliche
Proteine und lösliche oder nichtlösliche Fragmente davon,
die TNF binden (TNF-BP), in homogener Form, sowie deren
25 physiologisch verträgliche Salze. Bevorzugt sind solche
Proteine, die gemäss SDS-PAGE unter nicht reduzierenden
Bedingungen durch scheinbare Molekulargewichte von etwa
55 kD, 51 kD, 38 kD, 36 kD und 34 kD bzw. 75 kD und 65 kD
charakterisiert sind, insbesondere solche mit etwa 55 kD und
30 75 kD. Weiterhin bevorzugt sind solche Proteine, die durch
wenigstens eine der folgenden Aminosäureteilsequenzen
gekennzeichnet sind:

(IA) Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-
35 Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-
Ile

- (IB) Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys
- (IIA) Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu
- 5 (IIB) Val-Phe-Cys-Thr
- (IIC) Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala
- (IID) Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys
- 10 (IIE) Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu
- (IIF) Leu-Cys-Ala-Pro
- (IIG) Val-Pro-His-Leu-Pro-Ala-Asp
- (IIH) Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro

15 wobei X für einen Aminosäurerest steht, der nicht eindeutig bestimmt werden konnte.

Im Stand der Technik sind bereits TNF-BP durch eine N-terminale Teilsequenz charakterisiert worden [Europäische
20 Patentanmeldung mit der Publikations-Nr. 308 378], wobei sich diese Sequenz von der erfindungsgemässen N-terminalen Teilsequenz gemäss Formel (IA) unterscheidet. Im übrigen handelt es sich aber bei den im Stand der Technik beschriebenen TNF-Bindeproteinen um aus dem Urin isolierte,
25 lösliche, d.h. nicht membrangebundene, TNF-BP und nicht um membrangebundene, d.h. unlösliche, TNF-BP.

Gegenstand der vorliegenden Anmeldung sind auch Verfahren zur Isolierung der erfindungsgemässen TNF-BP. Diese
30 Verfahren sind dadurch charakterisiert, dass man im wesentlichen die folgenden Reinigungsschritte nacheinander ausführt: Herstellung eines Zell- oder Gewebeextraktes, Immunaффinitätschromatographie und/oder ein- oder mehrfache Ligandenaffinitätschromatographie, hochauflösende Flüssigkeitschromatographie (HPLC) und präparative SDS-Polyacrylamidgelelektrophorese (SDS-PAGE). Die Kombination der aus
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dem Stand der Technik bekannten einzelnen Reinigungsschritte ist für den Erfolg des erfindungsgemässen Verfahrens essentiell, wobei einzelne Schritte im Rahmen der zu lösenden Aufgabe modifiziert und verbessert wurden. So wurde
5 beispielsweise der ursprünglich für die Anreicherung von TNF-BP aus humaner Placenta [31] verwendete kombinierte Immunaффinitätschromatographie/TNF α -Ligandenaffinitätschromatographie-Schritt dadurch abgeändert, dass eine BSA-Sepharose 4B-Vorsäule verwendet wurde. Diese Vorsäule wurde zum
10 Auftrag des Zell- oder Membranextraktes in Reihe mit der Immunaффinitätssäule und gefolgt von der Ligandenaffinitätssäule geschaltet. Nach Auftrag des Extraktes wurden die beiden zuletztgenannten Säulen abgekoppelt, jede für sich eluiert und die TNF-BP-aktiven Fraktionen wurden nochmals
15 über eine Ligandenaffinitätssäule gereinigt. Erfindungswesentlich für die Durchführung des Umkehrphasen-HPLC-Schrittes ist die Verwendung eines Detergens-haltigen Lösungsmittelgemisches.

20 Ferner ist auch ein technisches Verfahren zum Erzielen hoher Zelldichten von Säugerzellen, aus denen TNF-BP isoliert werden können, Gegenstand der vorliegenden Erfindung. Ein solches Verfahren zeichnet sich dadurch aus, dass ein Medium, welches für die spezifischen Wachstumserfordernisse
25 der verwendeten Zelllinie entwickelt wurde, in Verbindung mit einer wie z.B. im Detail in Beispiel 2 beschriebenen Perfusionsapparatur verwendet wird. Mittels eines solchen Verfahrens lassen sich beispielsweise für HL-60-Zellen bis zu mehr als 20-fach höhere Zelldichten als üblich erzielen.

30 Zusätzlich dazu betrifft die vorliegende Erfindung auch DNA-Sequenzen, die für Proteine und lösliche oder nicht-lösliche Fragmente davon, die TNF binden, kodieren. Bevorzugt sind einmal DNA-Sequenzen, welche für ein solches
35 Protein mit einem scheinbaren Molekulargewicht von etwa 55 kD kodieren, wobei die in Abbildung 1 dargestellte Sequenz besonders bevorzugt ist, wie Sequenzen, die für nicht-

lösliche wie lösliche Fragmente von solchen Proteinen kodieren. Eine besonders bevorzugte DNA-Sequenz, die für ein nichtlösliches Protein-Fragment kodiert, reicht von Nukleotid -185 bis 1122 der in Abbildung 1 gezeigten Sequenz. Besonders bevorzugte DNA-Sequenzen, die für lösliche Protein-Fragmente kodieren, sind solche, die von Nukleotid -185 bis 633 bzw. von Nukleotid -14 bis 633 der in Abbildung 1 gezeigten Sequenz reichen. Bevorzugt sind ebenfalls DNA-Sequenzen, die für ein Protein von etwa 75/65 kD kodieren und die die in Figur 4 dargestellte partielle cDNA-Sequenzen enthalten. Besonders bevorzugte DNA-Sequenzen sind in diesem Fall die Sequenzen des offenen Leserasters von Nukleotid 2 bis 1'177. Die Peptide IIA, IIC, IIE, IIF, IIG und IIH werden von der partiellen cDNA-Sequenz in Figur 4 kodiert, wobei die geringfügigen Abweichungen der experimentell bestimmten Aminosäuresequenzen von der von der cDNA abgeleiteten Sequenz mit höchster Wahrscheinlichkeit auf der geringeren Auflösung der Gasphasen-Sequenzierung beruhen. Die vorliegende Erfindung betrifft natürlich auch die von solchen DNA-Sequenzen kodierten rekombinanten Proteine. Selbstverständlich sind dabei auch solche Proteine umfasst, in deren Aminosäuresequenzen, beispielsweise mittels gezielter Mutagenese, Aminosäuren so ausgetauscht worden sind, dass dadurch die Aktivität der TNF-BP oder deren Fragmente, nämlich die Bindung von TNF oder die Wechselwirkung mit anderen, an der Signalübertragung beteiligten Membrankomponenten, in einer gewünschten Art verändert oder erhalten wurden. Aminosäureaustausche in Proteinen und Peptiden, die im allgemeinen die Aktivität solcher Moleküle nicht verändern, sind im Stand der Technik bekannt und beispielsweise von H. Neurath und R.L. Hill in "The Proteins" (Academic Press, New York, 1979, siehe besonders Figur 6, Seite 14) beschrieben. Die am häufigsten vorkommenden Austausche sind: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly, sowie solche in umgekehrter Weise. Die

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vorliegende Erfindung betrifft ferner Vektoren, die erfindungsgemässe DNA-Sequenzen enthalten und zur Transformation von geeigneten pro- wie eukaryotischen Wirtssystemen geeignet sind, wobei solche Vektoren bevorzugt sind, deren Verwendung zur Expression der von den erfindungsgemässen DNA-Sequenzen kodierten Proteine führt. Schliesslich betrifft die vorliegende Erfindung auch noch mit solchen Vektoren transformierte pro- wie eukaryotische Wirtssysteme, wie Verfahren zur Herstellung von erfindungsgemässen rekombinanten Verbindungen durch Kultivierung solcher Wirtssysteme und anschliessende Isolierung dieser Verbindungen aus den Wirtssystemen selbst oder deren Kulturüberständen.

Gegenstand der vorliegenden Erfindung sind auch pharmazeutische Präparate, die wenigstens eines dieser TNF-BP oder Fragmente davon, gewünschtenfalls in Verbindung mit weiteren pharmazeutisch wirksamen Substanzen und/oder nicht-toxischen, inerten, therapeutisch verträglichen Trägermaterialien enthalten.

Die vorliegende Erfindung betrifft schliesslich die Verwendung solcher TNF-BP einerseits zur Herstellung pharmazeutischer Präparate bzw. andererseits zur Behandlung von Krankheiten, bevorzugt solchen, in deren Verlauf TNF involviert ist.

Ausgangsmaterial für die erfindungsgemässen TNF-BP sind ganz allgemein Zellen, die solche TNF-BP in membrangebundener Form enthalten und die dem Fachmann ohne Beschränkungen allgemein zugänglich sind, wie beispielsweise HL60- [ATCC Nr. CCL 240], U 937- [ATCC Nr. CRL 1593], SW 480- [ATCC Nr. CCL 228] und HEp2-Zellen [ATCC Nr. CCL 23]. Diese Zellen können nach bekannten Methoden des Standes der Technik [40] oder zum Erzielen hoher Zelldichten nach dem bereits allgemein und im Detail für HL60-Zellen in Beispiel 2 beschriebenen Verfahren kultiviert werden. TNF-BP können dann nach

bekannten Methoden des Standes der Technik mittels geeigneter Detergenzien, beispielsweise Triton X-114, 1-O-n-Octyl- β -D-glucopyranosid (Octylglucosid), oder 3-[(3-Cholylamidopropyl)-dimethylammonio]-1-propan sulfonat (CHAPS), im
5 besonderen mittels Triton X-100, aus den aus dem Medium abzentrifugierten und gewaschenen Zellen extrahiert werden. Zum Nachweis solcher TNF-BP können die üblicherweise verwendeten Nachweismethoden für TNF-BP, beispielsweise eine Poly-
10 äthylenglykol-induzierte Fällung des ^{125}I -TNF/TNF-BP-Komplexes [27], im besonderen Filterbindungstests mit radioaktiv markiertem TNF gemäss Beispiel 1, verwendet werden. Zur Gewinnung der erfindungsgemässen TNF-BP können die
15 generell zur Reinigung von Proteinen, insbesondere von Membranproteinen, verwendeten Methoden des Standes der Technik, wie beispielsweise Ionenaustausch-Chromatographie, Gelfiltration, Affinitätschromatographie, HPLC und SDS-PAGE
20 verwendet werden. Besonders bevorzugte Methoden zur Herstellung erfindungsgemässer TNF-BP sind Affinitätschromatographie, insbesondere mit TNF- α als an die Festphase gebundenen Liganden und Immunaффinitätschromatographie, HPLC
25 und SDS-PAGE. Die Elution von mittels SDS-PAGE aufgetrennten TNF-BP Banden kann nach bekannten Methoden der Proteinchemie erfolgen, beispielsweise mittels Elektroelution nach Hunkapiller et al. [34], wobei nach heutigem Stand des
Wissens die dort angegebenen Elektro-Dialysezeiten generell zu verdoppeln sind. Danach noch verbleibende Spuren von SDS können dann gemäss Bosserhoff et al. [50] entfernt werden.

Die so gereinigten TNF-BP können mittels der im Stand
30 der Technik bekannten Methoden der Peptidchemie, wie beispielsweise N-terminale Aminosäuresequenzierung oder enzymatische wie chemische Peptidspaltung charakterisiert werden. Durch enzymatische oder chemische Spaltung erhaltene
Fragmente können nach gängigen Methoden, wie beispielsweise
35 HPLC, aufgetrennt und selbst wieder N-terminal sequenziert werden. Solche Fragmente, die selbst noch TNF binden, können mittels der obengenannten Nachweismethoden für TNF-BP

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identifiziert werden und sind ebenfalls Gegenstand der vorliegenden Erfindung.

- Ausgehend von der so erhältlichen Aminosäuresequenz-
- 5 information oder den in Figur 1 dargestellten DNA- wie Aminosäuresequenzen können unter Beachtung der Degeneration des genetischen Codes nach im Stand der Technik bekannten Methoden geeignete Oligonukleotide hergestellt werden [51]. Mittels dieser können dann wiederum nach bekannten Methoden
- 10 der Molekularbiologie [42,43] cDNA- oder genomische DNA-Banken nach Klonen, die für TNF-BP kodierende Nukleinsäuresequenzen enthalten, abgesucht werden. Ausserdem können mittels der Polymerase-Kettenreaktion (PCR) [49] cDNA-Fragmente kloniert werden, indem von zwei auseinanderliegenden,
- 15 relativ kurzen Abschnitten der Aminosäuresequenz unter Beachtung des genetischen Codes vollständig degenerierte und in ihrer Komplementarität geeignete Oligonucleotide als "Primer" eingesetzt werden, wodurch das zwischen diesen beiden Sequenzen liegende Fragment amplifiziert und identifiziert werden kann. Die Bestimmung der Nukleotidsequenz
- 20 eines derartigen Fragmentes ermöglicht eine unabhängige Bestimmung der Aminosäure-Sequenz des Proteinfragments, für das es kodiert. Die mittels der PCR erhältlichen cDNA-Fragmente können ebenfalls, wie bereits für die Oligonukleotide selbst beschrieben, nach bekannten Methoden zum Aufsuchen
- 25 von für TNF-BP kodierende Nukleinsäuresequenzen enthaltenden Klonen aus cDNA- bzw. genomische DNA-Banken verwendet werden. Solche Nukleinsäuresequenzen können dann nach bekannten Methoden sequenziert werden [42]. Aufgrund der so
- 30 bestimmten wie der für bestimmte Rezeptoren bereits bekannten Sequenzen, können solche Teilsequenzen, die für lösliche TNF-BP-Fragmente kodieren, bestimmt und mittels bekannter Methoden aus der Gesamtsequenz herausgeschnitten werden [42].
- 35 Die gesamte Sequenz oder solche Teilsequenzen können dann mittels bekannter Methoden in im Stand der Technik beschriebene Vektoren zu deren Vervielfältigung wie Expres-

sion in Prokaryoten integriert werden [42]. Geeignete prokaryotische Wirtsorganismen stellen beispielsweise gram-negative wie gram-positive Bakterien, wie beispielsweise *E. coli* Stämme, wie *E. coli* HB 101 [ATCC Nr. 33 694] oder *E. coli* W3110 [ATCC Nr. 27 325] oder *B. subtilis* Stämme dar.

Weiterhin können erfindungsgemässe Nukleinsäuresequenzen, die für TNF-BP sowie für TNF-BP-Fragmente kodieren, in geeignete Vektoren zur Vermehrung wie Expression in eukaryotischen Wirtszellen, wie beispielsweise Hefe, Insekten- und Säugerzellen, mittels bekannter Methoden integriert werden. Expression solcher Sequenzen erfolgt bevorzugt in Säuger- wie Insektenzellen.

Ein typischer Expressionsvektor für Säugerzellen enthält ein effizientes Promotorelement, um eine gute Transkriptionsrate zu erzielen, die zu exprimierende DNA-Sequenz und Signale für eine effiziente Termination und Polyadenylierung des Transkripts. Weitere Elemente, die verwendet werden können, sind "Enhancer", welche zu nochmals verstärkter Transkription führen und Sequenzen, welche z.B. eine längere Halbwertszeit der mRNA bewirken können.

Die meisten Vektoren, die für eine transiente Expression einer bestimmten DNA-Sequenz in Säugerzellen verwendet werden, enthalten den Replikationsursprung des SV40 Virus. In Zellen, die das T-Antigen des Virus exprimieren, (z.B. COS-Zellen), werden diese Vektoren stark vermehrt. Eine vorübergehende Expression ist aber nicht auf COS-Zellen beschränkt. Im Prinzip kann jede transfektierbare Säugerzelllinie hierfür verwendet werden. Signale, die eine starke Transkription bewirken können, sind z.B. die frühen und späten Promotoren von SV40, der Promoter und Enhancer des "major immediate-early" Gens des HCMV (humaner Cytomegalovirus), die LTRs ("long terminal repeats") von Retroviren, wie beispielsweise RSV, HIV und MMTV. Es können aber auch Signale von zellulären Genen, wie z.B. die Promotoren des

Aktin- und Collagenase-Gens, verwendet werden.

Alternativ können aber auch stabile Zelllinien, die die spezifische DNA-Sequenz im Genom (Chromosom) integriert haben, erhalten werden. Hierzu wird die DNA-Sequenz zusammen mit einem selektierbaren Marker, z.B. Neomycin, Hygromycin, Dihydrofolat-Reduktase (dhfr) oder Hypoxanthin-Guanin-Phosphoribosyltransferase (hgpt) kotransfiziert. Die stabil ins Chromosom eingebaute DNA-Sequenz kann auch noch stark vermehrt werden. Ein geeigneter Selektionsmarker hierfür ist beispielsweise die Dihydrofolat-Reduktase (dhfr). Säugerzellen (z.B. CHO-Zellen), welche kein intaktes dhfr-Gen enthalten, werden hierbei nach erfolgter Transfektion mit steigenden Mengen von Methotrexat inkubiert. Auf diese Weise können Zelllinien erhalten werden, welche mehr als tausend Kopien der gewünschten DNA-Sequenz enthalten.

Säugerzellen, welche für die Expression verwendet werden können, sind z.B. Zellen der menschlichen Zelllinien Hela [ATCC CCL2] und 293 [ATCC CRL 1573], sowie 3T3- [ATCC CCL 163] und L-Zellen, z.B. [ATCC CCL 149], (CHO)-Zellen [ATCC CCL 61], BHK [ATCC CCL 10]-Zellen sowie die CV 1 [ATCC CCL 70]- und die COS-Zelllinien [ATCC CRL 1650, CRL 1651].

Geeignete Expressionsvektoren umfassen beispielsweise Vektoren wie pBC12MI [ATCC 67 109], pSV2dhfr [ATCC 37 146], pSVL [Pharmacia, Uppsala, Sweden], pRSVcat [ATCC 37 152] und pMSG [Pharmacia, Uppsala, Sweden]. Besonders bevorzugte Vektoren sind die in Beispiel 9 verwendeten Vektoren "pK19" und "pN123". Diese können aus den mit ihnen transformierten E. coli-Stämmen HB101(pK19) und HB101(pN123) nach bekannten Methoden isoliert werden [42]. Diese E. coli-Stämme wurden am 26. Januar 1990 bei der Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, BRD unter DSM 5761 für HB101(pK19) und DSM 5764 für HB101(pN123) hinterlegt.

Die Art und Weise wie die Zellen transfektiert werden hängt vom gewählten Expressions- und Vektorsystem ab. Eine Uebersicht über diese Methoden findet man z.B. bei Pollard et al., "DNA Transformation of Mammalian Cells" in "Methods in Molecular Biology" [Nucleic Acids Vol. 2, 1984, Walker, J.M., ed. Humana, Clifton, New Jersey]. Weitere Methoden findet man bei Chen und Okayama ["High-Efficiency Transformation of Mammalian Cells by Plasmid DNA", Molecular and Cell Biology 7, 2745-2752, 1987] und bei Felgner [Felgner et al., "Lipofectin: A highly efficient, lipid-mediated DNA-transfection procedure", Proc. Nat. Acad. Sci. USA 84, 7413-7417, 1987].

Zur Expression in Insektenzellen kann das Baculovirus-Expressions-System, welches schon für die Expression einer Reihe von Proteinen erfolgreich eingesetzt worden ist (für eine Uebersicht siehe Luckow and Summers, Bio/Technology 6, 47-55, 1988), verwendet werden. Rekombinante Proteine können authentisch oder als Fusionsproteine hergestellt werden. Die so hergestellten Proteine können auch modifiziert, wie beispielsweise glykosyliert (Smith et al., Proc. Nat. Acad. Sci. USA 82, 8404-8408, 1987) sein. Für die Herstellung eines rekombinanten Baculovirus, der das gewünschte Protein exprimiert, verwendet man einen sogenannten "Transfervektor". Hierunter versteht man ein Plasmid, welches die heterologe DNA-Sequenz unter der Kontrolle eines starken Promoters, z.B. dem des Polyhedringens, enthält, wobei diese auf beiden Seiten von viralen Sequenzen umgeben ist. Besonders bevorzugte Vektoren sind die in Beispiel 10 verwendeten Vektoren "pN113", "pN119" und "pN124". Diese können aus den mit ihnen transformierten E. coli-Stämmen HB101(pN113), HB101(pN119) und HB101(pN124) nach bekannten Methoden isoliert werden [42]. Diese E. coli-Stämme wurden am 26. Januar 1990 bei der Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, BRD, unter DSM 5762 für HB101(pN113), DSM 5763 für HB101(pN119) und DSM 5765 für HB101(pN124) hinterlegt. Der Transfervektor wird dann

- zusammen mit DNA des Wildtyp-Baculovirus in die Insektenzellen transfektiert. Die in den Zellen durch homologe Rekombination entstehenden rekombinanten Viren können dann nach bekannten Methoden identifiziert und isoliert werden.
- 5 Eine Uebersicht über das Baculovirus-Expressionssystem und der dabei verwendeten Methoden findet man bei Luckow und Summers [52].

- 10 Exprimierte TNF-BP wie ihre nichtlöslichen oder löslichen Fragmente können dann nach im Stand der Technik bekannten Methoden der Proteinchemie, wie beispielsweise den bereits auf Seiten 5-6 beschriebenen Verfahren, aus der Zellmasse oder den Kulturüberständen gereinigt werden.

- 15 Die erfindungsgemäss erhaltenen TNF-BP können auch als Antigene zur Erzeugung von poly- und monoklonalen Antikörpern nach bekannten Methoden der Technik [44,45] oder gemäss dem in Beispiel 3 beschriebenen Verfahren verwendet werden. Solche Antikörper, insbesondere monoklonale Antikörper gegen die 75 kD-TNF-BP-Spezies, sind ebenfalls Gegenstand der vorliegenden Erfindung. Solche gegen die 75 kD TNF-BP gerichtete Antikörper können durch dem Fachmann geläufige Modifikationen des in den Beispielen 4-6 im Detail beschriebenen Reinigungsverfahrens zur Isolierung von TNF-BP
- 20 eingesetzt werden.
- 25

- Auf Grund der hohen Bindungsaffinität erfindungsgemässer TNF-BP für TNF (K_d -Werte in den Grössenordnungen von 10^{-9} - 10^{-10} M) können diese oder Fragmente davon als
- 30 Diagnostika zum Nachweis von TNF in Serum oder anderen Körperflüssigkeiten nach im Stand der Technik bekannten Methoden, beispielsweise in Festphasenbindungstests oder in Verbindung mit Anti-TNF-BP-Antikörpern in sogenannten "Sandwich"-Tests, eingesetzt werden.
- 35

Im übrigen können erfindungsgemässe TNF-BP einerseits zur Reinigung von TNF und andererseits zum Auffinden von

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TNF-Agonisten sowie TNF-Antagonisten nach im Stand der Technik bekannten Verfahren verwendet werden.

Die erfindungsgemässen TNF-BP sowie deren physiologisch verträgliche Salze, die nach im Stand der Technik bekannten Methoden hergestellt werden können, können auch zur Herstellung von pharmazeutischen Präparaten, vor allem solchen zur Behandlung von Krankheiten, bei deren Verlauf TNF involviert ist, verwendet werden. Dazu kann eine oder mehrere der genannten Verbindungen, falls wünschenswert bzw. erforderlich in Verbindung mit anderen pharmazeutisch aktiven Substanzen, mit den üblicherweise verwendeten festen oder flüssigen Trägermaterialien in bekannter Weise verarbeitet werden. Die Dosierung solcher Präparate kann unter Berücksichtigung der üblichen Kriterien in Analogie zu bereits verwendeten Präparaten ähnlicher Aktivität und Struktur erfolgen.

Nachdem die Erfindung vorstehend allgemein beschrieben worden ist, sollen die folgenden Beispiele Einzelheiten der Erfindung veranschaulichen, ohne dass diese dadurch in irgendeiner Weise eingeschränkt wird.

Beispiel 1

25

Nachweis von TNF-bindenden Proteinen

Die TNF-BP wurden in einem Filtertest mit humanem radiojodiertem ^{125}I -TNF nachgewiesen. TNF (46,47) wurde mit ^{125}NaI (IMS40, Amersham, Amersham, England) und Iodo-Gen (#28600, Pierce Eurochemie, Oud-Beijerland, Niederlande) nach Fraker und Speck [48] radioaktiv markiert. Zum Nachweis der TNF-BP wurden isolierte Membranen der Zellen oder ihre solubilisierten, angereicherten und gereinigten Fraktionen auf angefeuchtete Nitrocellulose-Filter (0.45 μ , BioRad, Richmond, California, USA) aufgetragen. Die Filter wurden dann in Pufferlösung mit 1% entfettetem Milchpulver

blockiert und anschliessend mit $5 \cdot 10^5$ cpm/ml ^{125}I -TNF α ($0.3-1.0 \cdot 10^8$ cpm/ μg) in zwei Ansätzen mit und ohne Beigabe von $5 \mu\text{g/ml}$ nicht-markiertem TNF α inkubiert, gewaschen und luftgetrocknet. Die gebundene Radioaktivität wurde autoradiographisch semiquantitativ nachgewiesen oder in einem γ -Counter gezählt. Die spezifische ^{125}I -TNF- α -Bindung wurde nach Korrektur für unspezifische Bindung in Anwesenheit von unmarkiertem TNF- α im Ueberschuss ermittelt. Die spezifische TNF-Bindung im Filtertest wurde bei verschiedenen TNF-Konzentrationen gemessen und nach Scatchard analysiert [33], wobei ein K_d -Wert von $\sim 10^{-9}-10^{-10}$ M ermittelt wurde.

15

Beispiel 2

Zellextrakte von HL-60-Zellen

HL60 Zellen [ATCC-Nr. CCL 240] wurden in experimentellem Labormasstab in einem RPMI 1640-Medium [GIBCO-Katalog Nr. 074-01800], das noch 2 g/l NaHCO_3 und 5% fötales Kälberserum enthielt, in einer 5% CO_2 -Atmosphäre kultiviert und anschliessend zentrifugiert.

Zum Erzielen hoher Zelldichten in technischem Masstab wurde folgendermassen verfahren. Die Züchtung wurde in einem 75 l Airliftfermenter (Fa. Chemap, Schweiz) mit 58 l Arbeitsvolumen durchgeführt. Hierfür wurde das Kassettenmembransystem "PROSTAK" (Millipore, Schweiz) mit einer Membranfläche von $0,32 \text{ m}^2$ (1 Kassette) in den äusseren Zirkulationskreislauf integriert. Das Kulturmedium (siehe Tabelle 1) wurde mit einer Watson-Marlow Pumpe, Typ 603U, mit 5 l/min. umgepumpt. Nach einer Dampfsterilisation der Anlage, wobei das "PROSTAK" System im Autoklaven separat sterilisiert wurde, wurde die Fermentation mit wachsenden HL-60 Zellen aus einem 20 l Airliftfermenter (Chemap) gestartet. Die Zellzüchtung im Impffermenter erfolgte im

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- konventionellen Batchverfahren in dem Medium gemäss Tabelle 1 und einem Startzelltiter von 2×10^5 Zellen/ml. Nach 4 Tagen wurde der HL60 Ansatz mit einem Titer von 4.9×10^6 Zellen/ml in den 75 l Fermenter überführt. Der pH-Wert wurde bei 7.1 und der pO_2 Wert bei 25% Sättigung gehalten, wobei der Sauerstoffeintrag durch eine mikroporöse Fritte erfolgte. Nach anfänglicher Batchfermentation wurde am 2. Tag die Perfusion bei einem Zelltiter von 4×10^6 Zellen/ml mit 30 l Mediumsaustausch pro Tag gestartet. Auf der Filtratseite der Membran wurde das konditionierte Medium abgezogen und durch den Zulauf von frischem Medium ersetzt. Das Zulaufmedium wurde wie folgt verstärkt: Primatone von 0.25% auf 0.35%, Glutamin von 5 mM auf 6 mM und Glucose von 4 g/l auf 6 g/l. Die Perfusionsrate wurde dann am 3. und 4. Tag auf 72 l Medium/Tag und am 5. Tag auf 100 l Medium/Tag erhöht. Nach 120 Stunden der kontinuierlichen Züchtung wurde die Fermentation beendet. Unter den gegebenen Fermentationsbedingungen erfolgte exponentielles Zellwachstum bis 40×10^6 Zellen/ml. Die Verdopplungszeit der Zellpopulation betrug bis 10×10^6 Zellen/ml 20-22 Stunden und stieg dann mit zunehmender Zelldichte auf 30-36 Stunden an. Der Anteil der lebenden Zellen lag während der gesamten Fermentationszeit bei 90-95%. Der HL-60 Ansatz wurde dann im Fermenter auf ca. 12°C heruntergekühlt und die Zellen durch Zentrifugation (Beckman-Zentrifuge [Modell J-6B, Rotor JS], 3000 rpm, 10 min., 4°C) geerntet.

Tabelle 1

30 HL-60 Medium

Komponenten	Konzentrationen
	mg/l
$CaCl_2$ (wasserfrei)	112.644
$Ca(NO_3)_2 \cdot 4H_2O$	20
35 $CuSO_4 \cdot 5H_2O$	$0.498 \cdot 10^{-3}$
$Fe(NO_3)_3 \cdot 9H_2O$	0.02
$FeSO_4 \cdot 7H_2O$	0.1668

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	KCl	336,72
	KNO ₃	0,0309
	MgCl ₂ (wasserfrei)	11,444
	MgSO ₄ (wasserfrei)	68,37
5	NaCl	5801,8
	Na ₂ HPO ₄ (wasserfrei)	188,408
	NaH ₂ PO ₄ •H ₂ O	75
	Na ₂ SeO ₃ •5H ₂ O	9,6•10 ⁻³
	ZnSO ₄ •7H ₂ O	0,1726
10	D-Glucose	4000
	Glutathion (red.)	0,2
	Hepes-Puffer	2383,2
	Hypoxanthin	0,954
15	Linolsäure	0,0168
	Liponsäure	0,042
	Phenolrot	10,24
	Putrescin 2HCl	0,0322
	Na-Pyruvat	88
20	Thymidin	0,146
	Biotin	0,04666
	D-Ca-Pantothenat	2,546
	Cholinchlorid	5,792
	Folsäure	2,86
25	i-Inositol	11,32
	Niacinamid	2,6
	Nicotinamid	0,0074
	para-Aminobenzoessäure	0,2
	Pyridoxal HCl	2,4124
30	Pyridoxin HCl	0,2
	Riboflavin	0,2876
	Thiamin HCl	2,668
	Vitamin B ₁₂	0,2782
35	L-Alanin	11,78
	L-Asparaginsäure	10
	L-Asparagin H ₂ O	14,362

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	L-Arginin	40
	L-Arginin HCl	92,6
	L-Aspartat	33,32
	L-Cystin 2HCl	62,04
5	L-Cystein HCl·H ₂ O	7,024
	L-Glutaminsäure	36,94
	L-Glutamin	730
	L-Glycin	21,5
	L-Histidin	3
10	L-Histidin HCl·H ₂ O	27,392
	L-Hydroxyprolin	4
	L-Isoleucin	73,788
	L-Leucin	75,62
	L-Lysin HCl	102,9
15	L-Methionin	21,896
	L-Phenylalanin	43,592
	L-Prolin	26,9
	L-Serin	31,3
	L-Threonin	53
20	L-Tryptophan	11,008
	L-Tyrosin·2Na	69,76
	L-Valin	62,74
	Penicillin/Streptomycin	100 U/ml
25	Insulin (human)	5 µg/ml
	Transferrin (human)	15 µg/ml
	Rinderserumalbumin	67 µg/ml
	Primatec RL (Sheffield Products, Norwich NY, USA)	0,25%
30	Pluronic F68 (Serva, Heidelberg, BRD)	0,01%
	Fötales Kälberserum	0,3-3%

Das Zentrifugat wurde mit isotonem Phosphatpuffer (PBS;
 35 0,2 g/l KCl, 0,2 g/l KH₂PO₄, 8,0 g/l NaCl, 2,16 g/l
 Na₂HPO₄ · 7H₂O), der mit 5% Dimethylformamid, 10 mM
 Benzamidin, 100 E/ml Aprotinin, 10 µM Leupeptin, 1 µM

- Pepstatin, 1 mM o-Phenanthrolin, 5 mM Jodacetamid, 1 mM Phenylmethylsulfonylfluorid versetzt war (im folgenden als PBS-M bezeichnet), gewaschen. Die gewaschenen Zellen wurden bei einer Dichte von $2.5 \cdot 10^8$ Zellen/ml in PBS-M mit
- 5 Triton X-100 (Endkonzentration 1.0%) extrahiert. Der Zell-extrakt wurde durch Zentrifugation geklärt ($15'000 \times g$, 1 Stunde; $100'000 \times g$, 1 Stunde).

Beispiel 3

10

Herstellung von monoklonalen (TNF-BP)-Antikörpern

- Ein gemäss Beispiel 2 erhaltener Zentrifugationsüberstand aus Kultivierung von HL60-Zellen im experimentellen Labormasstab wurde im Verhältnis 1:10 mit PBS verdünnt. Der
- 15 verdünnte Ueberstand wurde bei 4°C auf eine Säule aufgetragen (Flussrate: 0.2 ml/min.), die 2 ml Affigel 10 enthielt (Bio Rad Katalog Nr. 153-6099), an das 20 mg rekombinantes humanes TNF- α [Pennica, D. et al. (1984) Nature
- 20 312, 724; Shirai, T. et al. (1985) Nature 313, 803; Wang, A.M. et al. (1985) Science 228, 149] gemäss den Empfehlungen des Herstellers gekoppelt worden war. Die Säule wurde bei 4°C und einer Durchflussrate von 1 ml/min zuerst mit 20 ml PBS, das 0.1% Triton X 114 enthielt und danach mit 20 ml PBS
- 25 gewaschen. So angereichertes TNF-BP wurde bei 22°C und einer Flussrate von 2 ml/min mit 4 ml 100 mM Glycin, pH 2.8, 0.1% Decylmaltosid eluiert. Das Eluat wurde in einer Centricon 30 Einheit [Amicon] auf 10 μ l konzentriert.

- 30 10 μ l dieses Eluates wurden mit 20 μ l vollständigem Freundschens Adjuvans zu einer Emulsion gemischt. Je 10 μ l der Emulsion wurden gemäss dem von Holmdahl, R. et al. [(1985), J. Immunol. Methods 83, 379] beschriebenen Verfahren an den Tagen 0, 7 und 12 in eine hintere Fusspfote
- 35 einer narkotisierten Balb/c-Maus injiziert.

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- Am Tag 14 wurde die immunisierte Maus getötet, der popliteale Lymphknoten herausgenommen, zerkleinert und in Iscove's Medium (IMEM, GIBCO Katalog Nr. 074-2200), das 2 g/l NaHCO_3 enthielt, durch wiederholtes Pipettieren suspendiert. Gemäss einem modifizierten Verfahren von De St.Groth und Scheidegger [J. Immunol. Methods (1980), 35, 1] wurden 5×10^7 Zellen des Lymphknotens mit 5×10^7 PAI Maus-Myelomazellen (J.W. Stocker et al., Research Disclosure, 217, Mai 1982, 155-157), die sich in logarithmischem Wachstum befanden, fusioniert. Die Zellen wurden gemischt, durch Zentrifugation gesammelt und durch leichtes Schütteln in 2 ml 50% (v/v) Polyethylenglycol in IMEM bei Raumtemperatur resuspendiert und durch langsame Zugabe von 10 ml IMEM während 10 Minuten vorsichtigen Schüttelns verdünnt. Die Zellen wurden durch Zentrifugation gesammelt und in 200 ml vollständigem Medium [IMEM + 20% fötales Kälberserum, Glutamin (2,0 mM), 2-Mercaptoethanol (100 μM), 100 μM Hypoxanthine, 0,4 μM Aminopterin und 16 μM Thymidine (HAT)] resuspendiert. Die Suspension wurde auf 10 Gewebekulturschalen, die jeweils 96 Vertiefungen enthielten, verteilt und ohne Wechsel des Mediums bei 37°C in einer Atmosphäre von 5% CO_2 und einer relativen Luftfeuchtigkeit von 98% 11 Tage lang inkubiert.
- Die Antikörper zeichnen sich aus durch ihre inhibierende Wirkung auf die TNF-Bindung an HL60-Zellen oder durch ihre Bindung an Antigen im Filtertest gemäss Beispiel 1. Zum Nachweis der biologischen Aktivität von anti(TNF-BP)-Antikörpern wurde folgendermassen verfahren: 5×10^6 HL60 oder U937-Zellen wurden in vollständigem RPMI 1640 Medium zusammen mit affinitätsgereinigten monoklonalen anti-(TNF-BP)-Antikörpern oder Kontrollantikörpern (d.h. solchen, die nicht gegen TNF-BP gerichtet sind) in einem Konzentrationsbereich von 1 ng/ml bis 10 $\mu\text{g/ml}$ inkubiert. Nach einer Stunde Inkubation bei 37°C wurden die Zellen durch Zentrifugation gesammelt und mit 4,5 ml PBS bei 0°C gewaschen. Sie wurden in 1 ml vollständigem RPMI 1640 Medium

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- (Beispiel 2), das zusätzlich 0,1% Natriumazid und ^{125}I -TNF α (10^6 cpm/ml) mit oder ohne Beigabe von unmarkiertem TNF α (s.o.) enthielt, resuspendiert. Die spezifische Radioaktivität des ^{125}I -TNF α betrug 700 Ci/mmol.
- 5 Die Zellen wurden 2 Stunden bei 4°C inkubiert, gesammelt und 4 mal mit 4,5 ml PBS, das 1% BSA und 0,001% Triton X 100 (Fluka) enthielt, bei 0°C gewaschen. Die an die Zellen gebundene Radioaktivität wurde in einem γ -Scintillationszähler gemessen. In einem vergleichbaren Experiment wurde
- 10 die zellgebundene Radioaktivität von Zellen, die nicht mit anti-(TNF-BP)-Antikörpern behandelt worden waren, bestimmt (ungefähr 10 000 cpm/ 5×10^6 Zellen).

Beispiel 4

15

Affinitätschromatographie

- Für die weitere Reinigung wurden jeweils ein gemäss Beispiel 3 erhaltener monoklonaler anti-(55 kD TNF-BP)-Antikörper (2,8 mg/ml Gel), TNF α (3,0 mg/ml Gel) und Rinderserumalbumin (BSA, 8,5 mg/ml Gel) gemäss den Vorschriften des Herstellers kovalent an CNBr-aktivierte Sepharose 4B (Pharmacia, Uppsala, Schweden) gekoppelt. Der gemäss
- 20 Beispiel 2 erhaltene Zellextrakt wurde über die so hergestellten und in der folgenden Reihenfolge hintereinandergeschalteten Säulen geleitet: BSA-Sepharose-Vorsäule, Immunaффinitätssäule [Anti-(55 kD-TNF-BP)-Antikörper], TNF α -Ligand-Affinitätssäule. Nach vollständigem Auftrag wurden die beiden letztgenannten Säulen abgetrennt und einzeln für
- 30 sich mit je 100 ml der folgenden Pufferlösungen gewaschen: (1) PBS, 1,0% Triton X-100, 10 mM Benzamidin, 100 E/ml Aprotinin; (2) PBS, 0,1% Triton X-100, 0,5M NaCl, 10 mM ATP, 10 mM Benzamidin, 100 E/ml Aprotinin; und (3) PBS, 0,1% Triton X-100, 10 mM Benzamidin, 100 E/ml Aprotinin. Sowohl
- 35 die Immun- als auch die TNF α -Ligand-Affinitätssäule wurden dann mit 100 mM Glycin pH 2,5, 100 mM NaCl, 0,2% Decylmaltoside, 10 mM Benzamidin, 100 E/ml Aprotinin jede für

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sich eluiert. Die im Filtertest gemäss Beispiel 1 aktiven Fraktionen jeder Säule wurden danach jeweils vereint und mit 1M Tris pH 8,0 neutralisiert.

- 5 Die so vereinten TNF-BP-aktiven Fraktionen der Immun-Affinitätschromatographie einerseits und der TNF α -Ligand-Affinitätschromatographie andererseits wurden zur weiteren Reinigung nochmals auf je eine kleine TNF α -Ligand-Affinitätssäule aufgetragen. Danach wurden diese beiden Säulen mit
- 10 je 40 ml von (1) PBS, 1,0% Triton X-100, 10 mM Benzamidin, 100 E/ml Aprotinin, (2) PBS, 0,1% Triton X-100, 0,5M NaCl, 10 mM ATP, 10mM Benzamidin, 100 E/ml Aprotinin, (3) PBS, 0,1% Triton X-100, (4) 50 mM Tris pH 7.5, 150 mM NaCl, 1,0% NP-40, 1,0% Desoxycholat, 0,1% SDS, (5) PBS, 0,2% Decyl-
- 15 maltosid gewaschen. Anschliessend wurden die Säulen mit 100 mM Glycin pH 2.5, 100 mM NaCl, 0,2% Decylmaltosid eluiert. Fraktionen von 0.5 ml von jeder Säule wurden für sich gesammelt und die gemäss Filtertest (Beispiel 1) aktiven Fraktionen von jeder Säule jeweils für sich vereint
- 20 und in einer Centricon-Einheit (Amicon, Molekulargewichts-Ausschluss 10'000) aufkonzentriert.

Beispiel 5

25 Auftrennung mittels HPLC

- Die gemäss Beispiel 4 erhaltenen aktiven Fraktionen wurden gemäss ihrer unterschiedlichen Herkunft (Immun- bzw. Ligand-Affinitätschromatographie) jeweils für sich auf C1/C8
- 30 Umkehrphasen-HPLC-Säulen (ProRPC, Pharmacia, 5x20 mm), die mit 0,1% Trifluoressigsäure, 0,1% Octylglucosid equilibriert worden waren, aufgetragen. Die Säulen wurden dann mit einem linearen Acetonitril-Gradienten (0-80%) im gleichen Puffer bei einem Fluss von 0.5 ml/min eluiert. Fraktionen von
- 35 1.0 ml wurden von jeder Säule gesammelt und die aktiven Fraktionen von jeder Säule für sich vereint (Nachweis gemäss Beispiel 1).

Beispiel 6

Auftrennung mittels SDS-PAGE

5 Die gemäss Beispiel 5 erhaltenen und gemäss Filtertest
(Beispiel 1) aktiven Fraktionen wurden durch SDS-PAGE gemäss
[34] weiter aufgetrennt. Dazu wurden die Proben in SDS-
-Probenpuffer während 3 Minuten auf 95°C erhitzt und
anschliessend auf einem 12% Acrylamid-Trenngel mit einem
10 5%igen Sammelgel elektrophoretisch aufgetrennt. Als Referenz
zur Bestimmung der scheinbaren Molekulargewichte auf dem
SDS-PAGE Gel wurden die folgenden Eichproteine verwendet:
Phosphorylase B (97,4 kD), BSA (66,2 kD), Ovalbumin
(42,7 kD), Carboanhydrase (31,0 kD), Soya Trypsin-Inhibitor
15 (21,5 kD) und Lysozym (14,4 kD).

Unter den genannten Bedingungen wurden für Proben, die
gemäss Beispiel 4 durch TNF- α -Ligandenaffinitätschromato-
graphie von Immunaaffinitätschromatographieeluat erhalten
20 und durch HPLC gemäss Beispiel 5 weiter aufgetrennt worden
waren, zwei Banden von 55 kD und 51 kD sowie drei schwächere
Banden von 38 kD, 36 kD und 34 kD erhalten. Diese Banden
wurden in einem Mini Trans Blot System (BioRad, Richmond,
California, USA) elektrophoretisch während 1 Stunde bei
25 100 V in 25 mM Tris, 192 mM Glycin, 20% Methanol auf eine
PVDF-Membran (Immobilon, Millipore, Bedford, Mass. USA)
transferiert. Danach wurde die PVDF-Membran entweder mit
0,15% Serva-Blau (Serva, Heidelberg, BRD) in Methanol/Was-
ser/Eisessig (50/40/10 Volumenteile) auf Protein gefärbt
30 oder mit entfettetem Milchpulver blockiert und anschliessend
zum Nachweis von Banden mit TNF-BP-Aktivität mit ^{125}I -
-TNF α gemäss den in Beispiel 1 beschriebenen Filtertest-
bedingungen inkubiert. Dabei zeigte sich, dass alle in der
Proteinfärbung zur Darstellung gelangten Banden spezifisch
35 TNF α banden. Alle diese Banden banden im Western Blot nach
Towbin et al. [38] auch den gemäss Beispiel 3 hergestellten
monoklonalen Anti-55kD-TNF-BP-Antikörper. Dabei wurde ein

gemäss dem in Beispiel 1 beschriebenen Verfahren mit
 Na^{125}I radioaktiv markierter, affinitätsgereinigter
 (Mausimmunglobulin-Sephrose-4B-Affinitätssäule)
 Kaninchen-anti-Maus-Immunglobulin-Antikörper zum
 5 autoradiographischen Nachweis dieses Antikörpers eingesetzt.

Proben, die gemäss Beispiel 4 durch zweimalige TNF- α -
 -Ligandenaffinitätschromatographie des Durchlaufs der Immun-
 affinitätschromatographie erhalten und durch HPLC gemäss
 10 Beispiel 5 weiter aufgetrennt worden waren, zeigten unter
 den oben spezifizierten SDS-PAGE- und Blottransfer-Bedingungen
 zwei zusätzliche Banden von 75 kD und 65 kD, die
 beide im Filtertest (Beispiel 1) spezifisch TNF banden. Im
 Western Blot gemäss Towbin et al. (s.o.) reagierten die
 15 Proteine dieser beiden Banden nicht mit dem gemäss Beispiel
 3 hergestellten anti-(55 kD TNF-BP)-Antikörper. Sie reagierten
 allerdings mit einem monoklonalen Antikörper, der
 ausgehend von der 75 kD-Bande (anti-75 kD TNF-BP-Antikörper)
 gemäss Beispiel 3 erzeugt worden war.

20

Beispiel 7

Aminosäuresequenzanalyse

25 Zur Aminosäuresequenzanalyse wurden die gemäss Beispiel
 5 erhaltenen und gemäss Filtertest (Beispiel 1) aktiven
 Fraktionen mittels der in Beispiel 6 beschriebenen, nun
 jedoch reduzierenden, SDS-PAGE Bedingungen (SDS-Probenpuffer
 mit 125 mM Dithiothreitol) aufgetrennt. Es wurden die
 30 gleichen Banden wie gemäss Beispiel 6 gefunden, die allerdings
 auf Grund der reduzierenden Bedingungen der SDS-PAGE
 im Vergleich zu Beispiel 6 alle um etwa 1-2 kD höhere
 Molekulargewichte zeigten. Diese Banden wurden dann gemäss
 Beispiel 6 auf PVDF-Membranen übertragen und mit 0,1%
 35 Serva-Blau in Methanol/Wasser/Eisessig (50/40/10 Volumenteile)
 während 1 Minute gefärbt, mit Methanol/Wasser/Eisessig
 (45/48/7 Volumenteile) entfärbt, mit Wasser gespült,

- luftgetrocknet und danach ausgeschnitten. Bei sämtlichen Schritten wurden zur Vermeidung von N-terminaler Blockierung die von Hunkapiller [34] angegebenen Bedingungen eingehalten. Zunächst wurden die gereinigten TNF-BP unverändert zur Aminosäuresequenzierung eingesetzt. Um zusätzliche Sequenzinformation zu erhalten, wurden die TNF-BP nach Reduktion und S-Carboxymethylierung [Jones, B.N. (1986) in "Methods of Protein Microcharacterisation", J.E. Shively, ed., Humana Press, Clifton NJ, 124-125] mit Bromcyan (Tarr, G.E. in "Methods of Protein Microcharacterisation", 165-166, op.cit.), Trypsin und/oder Proteinase K gespalten und die Peptide mittels HPLC nach bekannten Methoden der Proteinchemie aufgetrennt. So vorbereitete Proben wurden dann in einem automatisierten Gasphasen-Mikrosequenzier-Gerät (Applied Biosystems Modell 470A, ABI, Foster City, Calif., USA) mit einem on-line nachgeschalteten automatisierten HPLC PTH-Aminosäureanalysator (Applied Biosystems Modell 120, ABI s.o.) sequenziert, wobei die folgenden Aminosäuresequenzen bestimmt wurden:
- 1., Für die 55 kD-Bande (gemäß nichtreduzierender SDS-PAGE):
 Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile,
 und
 Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys
 wobei X für einen Aminosäurerest steht, der nicht bestimmt werden konnte.
 - 2., Für die 51 kD und die 38 kD-Banden (gemäß nichtreduzierender SDS-PAGE):
 Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu
 - 3., Für die 65 kD-Bande (gemäß nichtreduzierender SDS-PAGE):
 Bei der N-terminalen Sequenzierung der 65 kD Bande wurden bis zum 15. Rest ohne Unterbrechung zwei parallele Sequenzen ermittelt. Da eine der beiden

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Sequenzen einer Teilsequenz des Ubiquitins [36,37] entsprach, wurde für die 65 kD-Bande die folgende Sequenz abgeleitet:

5 Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys.

wobei X für einen Aminosäurerest steht, der nicht bestimmt werden konnte.

10 Weitere Peptidsequenzen für 75(65)kDa-TNF-BP wurden bestimmt:

Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu
und

15 Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu

und

Val-Phe-Cys-Thr

und

20 Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala

und

Leu-Cys-Ala-Pro

und

25 Val-Pro-His-Leu-Pro-Ala-Asp

und

Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro

30 wobei X für einen Aminosäurerest steht, der nicht bestimmt werden konnte.

Beispiel 8

Bestimmung von Basen-Sequenzen von komplementärer DNA (cDNA)

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Ausgehend von der Aminosäuresequenz gemäss Formel IA wurden unter Berücksichtigung des genetischen Codes zu den

Aminosäureresten 2-7 und 17-23 entsprechende, vollständig degenerierte Oligonucleotide in geeigneter Komplementarität synthetisiert ("sense" and "antisense" Oligonucleotide). Totale zelluläre RNA wurde aus HL60-Zellen isoliert [42, 43], und der erste cDNA-Strang durch Oligo-dT-Priming oder durch Priming mit dem "antisense" Oligonucleotid mittels eines cDNA-Synthese-Kits (RPN 1256, Amersham, Amersham, England) gemäss der Anleitung des Herstellers synthetisiert. Dieser cDNA-Strang und die beiden synthetisierten degenerierten "sense" und "anti-sense" Oligonucleotide wurden in einer Polymerase-Kettenreaktion (PCR, Perkin Elmer Cetus, Norwalk, CT, USA gemäss Anleitung des Herstellers) dazu verwendet, die für die Aminosäure-Reste 8-16 (Formel IA) codierende Basesequenz als cDNA-Fragment zu synthetisieren.

Die Basensequenz dieses cDNA-Fragmentes lautet:
 5'-AGGGAGAAGAGAGATAGTGTGTGTCCC-3'. Dieses cDNA-Fragment wurde als Probe verwendet, um nach bekannten Verfahren einen für das 55 kD TNF-BP codierenden cDNA-Klon in einer λ gt11-cDNA-Genbank von menschlicher Placenta zu identifizieren (42,43). Dieser Klon wurde dann nach üblichen Methoden aus dem λ -Vektor geschnitten und in die Plasmide pUC18 (Pharmacia, Uppsala, Sweden) und pUC19 (Pharmacia, Uppsala, Sweden) und in die M13mpl8/M13mpl9 Bacteriophagen (Pharmacia, Uppsala, Sweden) kloniert (42,43). Die Nukleotidsequenz dieses cDNA-Klons wurde mit einem Sequenase-Kit (U.S. Biochemical, Cleveland, Ohio, USA) nach den Angaben des Herstellers bestimmt. Die Nukleotidsequenz und die daraus abgeleitete Aminosäuresequenz für das 55 kD TNF-BP und dessen Signalpeptid (Aminosäure "-28" bis Aminosäure "0") ist in Figur 1 mittels der im Stand der Technik üblichen Abkürzungen für Basen wie Aminosäuren dargestellt. Aus Sequenzvergleichen mit anderen, bereits bekannten Rezeptorproteinsequenzen lassen sich ungefähr 180 Aminosäuren enthaltende N-terminale wie 220 Aminosäuren enthaltende C-terminale Domänen, die von einer nach den Sequenzvergleichen typischen Transmembran-Region von 19 Aminosäuren (in Figur 1 unterstrichen) getrennt werden.

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bestimmen. Hypothetische Glykosylierungsstellen sind in Figur 1 durch Sterne über der entsprechenden Aminosäure gekennzeichnet.

- 5 Im Wesentlichen analoge Techniken wurden dazu eingesetzt. 75/65 kD TNF-BP codierende partielle cDNA-Sequenzen zu identifizieren, wobei allerdings in diesem Fall genomische humane DNA und von Peptid IIA abgeleitete, vollständig degenerierte 14-mere und 15-mere "sense" und
10 "antisense" Oligonucleotide verwendet wurden, um eine primäre, 26 bp cDNA-Probe in einer Polymerase-Kettenreaktion herzustellen. Diese cDNA-Probe wurde dann dazu verwendet, in einer HL-60 cDNA-Bibliothek cDNA-Klone von verschiedener Länge zu identifizieren. Diese cDNA-Bibliothek
15 wurde mittels isolierter HL60 RNA und einem cDNA-Klonierungskit (Amersham) nach den Angaben des Herstellers hergestellt. Partielle Sequenzen dieser cDNA-Klone sind in Figur 4 dargestellt.

20

Beispiel 9

Expression in COS 1-Zellen

- Für die Expression in COS-Zellen wurden Vektoren
25 ausgehend von dem Plasmid "pN11" konstruiert. Das Plasmid "pN11" enthält den effizienten Promotor und Enhancer des "major immediate-early" Gens des menschlichen Cytomegalovirus ("HCMV"; Boshart et al., Cell 41, 521-530, 1985). Hinter dem Promotor befindet sich eine kurze DNA-Sequenz,
30 welche mehrere Restriktionsschnittstellen enthält, die nur einmal im Plasmid vorkommen ("Polylinker"), u.a. die Schnittstellen für HindIII, BalI, BamHI und PvuII (siehe Sequenz).

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PvuII

5' - AAGCTTGGCCAGGATCCAGCTGACTGACTGATCGCGAGATC - 3'
3' - TTCGAACCGGTCCTAGGTCGACTGACTGACTAGCGCTCTAG - 5'

Hinter diesen Schnittstellen befinden sich drei Translations-Stopcodons in allen drei Leserastern. Hinter der Polylinkersequenz befindet sich das 2. Intron und das Polyadenylierungssignal des Präproinsulins der Ratte (Lomedico et al., Cell 18, 545-558, 1979). Das Plasmid enthält ferner den Replikationsursprung des SV40 Virus sowie ein Fragment aus pBR322, das E. coli-Bakterien Ampicillin-Resistenz verleiht und die Replikation des Plasmids in E. coli ermöglicht.

Zur Konstruktion des Expressionsvektors "pN123" wurde dieses Plasmid "pN11" mit der Restriktionsendonuklease PvuII geschnitten und anschliessend mit alkalischer Phosphatase behandelt. Der dephosphorylierte Vektor wurde danach aus einem Agarosegel isoliert (V1). Die 5'-überhängenden Nukleotide des EcoRI-geschnittenen 1,3kb-Fragments der 55 kD TNF-BP-cDNA (siehe Beispiel 8) wurden mit Hilfe von Klenow-Enzym aufgefüllt. Anschliessend wurde dieses Fragment aus einem Agarosegel isoliert (F1). Danach wurden V1 und F1 mittels T4-Ligase miteinander verbunden. E. coli HB101-Zellen wurden dann mit diesem Ligierungsansatz nach bekannten Methoden [42] transformiert. Mit Hilfe von Restriktionsanalysen und DNA-Sequenzierung nach bekannten Methoden [42] wurden Transformanten identifiziert, die mit einem Plasmid transformiert worden waren, welches das 1,3kb EcoRI-Fragment der 55 kD TNF-BP-cDNA in der für die Expression über den HCMV-Promotor korrekten Orientierung enthielt. Dieser Vektor erhielt die Bezeichnung "pN123".

Zur Konstruktion des Vektors "pK19" wurde folgendermassen verfahren. Ein DNA-Fragment, welches nur die für den extrazellulären Teil des 55 kD TNF-BP codierende cDNA enthält (Aminosäuren -28 bis 182 gemäss Figur 1) wurde mittels PCR-Technologie erhalten (Saiki et al., Science 230, 1350-1354, 1985, siehe auch Beispiel 8). Die folgenden Oligonukleotide wurden, um die für den extrazellulären Teil des 55 kD TNF-BP codierende cDNA aus "pN123" zu amplifizieren, verwendet:

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BAMHI

5'-CACAGGGATCCATAGCTGTCTGGCATGGGCCTCTCCAC-3'

ASP718

5 3'-CGTGACTCCTGAGTCCGTGGTGTATTATCTCTAGACCATGGCCC-5'

Durch diese Oligonukleotide wurden ebenfalls zwei Stopkodons der Translation hinter Aminosäure 182 eingeführt. Das so amplifizierte DNA-Fragment wurde mit BamHI und Asp718 geschnitten, die hierbei entstandenen überstehenden Enden mit Hilfe des Klenow-Enzyms aufgefüllt und dieses Fragment anschliessend aus einem Agarosegel isoliert (F2). F2 wurde dann mit V1 ligiert und der gesamte Ansatz zur Transformation von E. coli HB101, wie bereits beschrieben, verwendet. Transformanten, die mit einem Plasmid transformiert worden waren, welches das DNA-Fragment in der für die Expression über den HCMV-Promotor korrekten Orientierung enthielten, wurden mittels DNA-Sequenzierung (s.o.) identifiziert. Das daraus isolierte Plasmid erhielt die Bezeichnung "pK19".

20 Transfektion der CQS-Zellen mit den Plasmiden "pN123" oder "pK19" wurde nach der von Felgner et al. veröffentlichten Lipofektions-Methode (Proc. Natl. Acad. Sci. USA 84, 7413-7417, 1987) durchgeführt. 72 Stunden nach erfolgter Transfektion wurden die mit "pN123" transfizierten Zellen nach bekannten Methoden mit ¹²⁵I-TNFα auf Bindung analysiert. Das Resultat der Scatchard-Analyse [Scatchard, G., Ann. N.Y. Acad. Sci. 51, 660, 1949] der so erhaltenen Bindungsdaten (Figur 2A) ist in Figur 2B dargestellt. Die Kulturüberstände der mit "pK19" transfizierten Zellen wurden in einem "Sandwich"-Test untersucht. Dazu wurden PVC-Microtiterplatten (Dynatech, Arlington, VA, USA) mit 100 µl/Loch eines Kaninchen-anti-Maus Immunglobulins (10 µg/ml PBS) sensibilisiert. Anschliessend wurde die Platte gewaschen und mit einem anti-55 kD TNF-BP-Antikörper, der gemäss Beispiel 3 durch seine Antigenbindung nachgewiesen und isoliert wurde, der aber die TNF-Bindung an

Zellen nicht inhibiert, inkubiert (3 Stunden, 20°C). Die Platte wurde dann wieder gewaschen und über Nacht bei 4°C mit 100 µl/Loch der Kulturüberstände (1:4 verdünnt mit 1% entfetteter Milchpulver enthaltendem Puffer A: 50 mM Tris/HCl pH 7.4, 140 mM NaCl, 5 mM EDTA, 0.02% Na-Azid) inkubiert. Die Platte wurde entleert und mit ¹²⁵I-TNFα enthaltendem Puffer A (10⁶ cpm/ml, 100 µl/Loch) mit oder ohne Zusatz von 2 µg/ml unmarkiertem TNF während 2 Stunden bei 4°C inkubiert. Danach wurde die Platte 4 mal mit PBS gewaschen, die einzelnen Löcher wurden ausgeschnitten und in einem γ-Zähler gemessen. Die Resultate von 5 parallelen Transfektionen (Säulen # 2, 3, 4, 6 und 7), von zwei Kontroll-Transfektionen mit dem pN11-Vektor (Säulen # 1, 5) und von einer Kontrolle mit HL60-Zell-Lysat (Säule # 8) sind in Figur 3 dargestellt.

Beispiel 10

Expression in Insektenzellen

Für die Expression in einem Baculovirus-Expressionssystem wurde von dem Plasmid "pVL941" (Luckow und Summers, 1989, "High Level Expression of Nonfused Foreign Genes with Autographa californica Nuclear Polyhedrosis virus Expression Vectors", Virology 170, 31-39) ausgegangen und dieses folgendermassen modifiziert. Es wurde die einzige EcoRI-Restriktionsschnittstelle in "pVL941" entfernt, indem das Plasmid mit EcoRI geschnitten und die überstehenden 5'-Enden mit Klenow-Enzym aufgefüllt wurden. Das hieraus erhaltene Plasmid pVL941/E- wurde mit BamHI und Asp718 verdaut und der Vektorrumpf anschliessend aus einem Agarosegel isoliert. Dieses Fragment wurde mit einem synthetischen Oligonukleotid der folgenden Sequenz ligiert:

35	BamHI EcoRI Asp718
	5' - GATCCAGAATTCATAATAG - 3'
	3' - GTCTTAAGTATTATCCATG - 5'

E. coli HB101 wurde mit dem Ligierungsansatz transformiert und Transformanten, die ein Plasmid enthielten, in welches das Oligonukleotid korrekt eingebaut worden war, wurden durch Restriktionsanalyse und DNA-Sequenzierung nach bekannten Methoden (s.o.) identifiziert; dieses Plasmid wurde "pNR704" genannt. Zur Konstruktion des Transfervektors "pN113" wurde dieses Plasmid "pNR704" mit EcoRI geschnitten, mit alkalischer Phosphatase behandelt und der so erzeugte Vektorrumpf (V2) anschliessend aus einem Agarosegel isoliert. Das wie oben mit EcoRI geschnittene 1,3 kb-Fragment der 55 kD TNF-BP-cDNA wurde mit Fragment V2 ligiert. Mit diesem Ligierungsansatz erhaltene Transformanten, die ein Plasmid enthielten, welches das cDNA-Insert in der korrekten Orientierung für die Expression über den Polyhedrinpromotor enthielten, wurden identifiziert (s.o.). Der daraus isolierte Vektor erhielt die Bezeichnung "pN113".

Zur Konstruktion des Transfervektors "pN119" wurde folgendermassen vorgegangen. Das 1,3 kb EcoRI/EcoRI-Fragment der 55 kD TNF-BP cDNA in dem "pUC19"-Plasmid (siehe Beispiel 8) wurde mit BanI verdaut und mit dem folgenden synthetischen Oligonukleotid ligiert:

	BanI	Asp718
25	5' - GCACCACATAATAGAGATCTGGTACCGGGAA - 3'	
	3' - GTGTATTATCTCTAGACCATGGCCC - 5'	

Mit dem obigen Adaptor werden zwei Stopcodons der Translation hinter Aminosäure 182 und eine Schnittstelle für die Restriktionsendonuklease Asp718 eingebaut. Nach erfolgter Ligation wurde der Ansatz mit EcoRI und Asp718 verdaut und das partielle 55 kD TNF-BP-Fragment (F3) isoliert. Weiterhin wurde das ebenfalls mit Asp718 und EcoRI geschnittene Plasmid "pNR704" mit F3 ligiert und der Ligierungsansatz in E. coli HB101 transformiert. Die Identifikation der Transformanten, welche ein Plasmid enthielten, in das die partielle 55 kD TNF-BP cDNA korrekt für die Expres-

sion integriert worden war, erfolgte wie bereits beschrieben. Das aus diesen Transformanten isolierte Plasmid erhielt den Namen "pN119".

- 5 Zur Konstruktion des Transfervektors "pN124" wurde folgendermassen vorgegangen. Das in Beispiel 9 beschriebene, für den extrazellulären Teil des 55 kD TNF-BP codierende cDNA-Fragment wurde mit den angegebenen Oligonukleotiden mit Hilfe der PCR-Technologie, wie in Beispiel 9 beschrieben, 10 amplifiziert. Dieses Fragment wurde mit BamHI und Asp718 geschnitten und aus einem Agarosegel isoliert (F4). Das Plasmid "pNR704" wurde ebenfalls mit BamHI und Asp718 geschnitten und der Vektorrumpf (V4) wurde isoliert (s.o.). Die Fragmente V4 und F4 wurden ligiert. E. coli HB101 damit 15 transformiert und der rekombinante Transfervektor "pN124" wurde, wie beschrieben, identifiziert und isoliert.

- Zur Transfektion der Insektenzellen wurde folgendermassen vorgegangen. 3 µg des Transfervektors "pN113" 20 wurden mit 1 µg DNA des Autographa californica-Nukleopolyhedrosisvirus (AcMNPV) (EP 127839) in Sf9-Zellen (ATCC CRL 1711) transfektiert. Polyhedrin negative Viren wurden identifiziert und aus "Plaques" gereinigt [52]. Mit diesen rekombinanten Viren wurden wiederum Sf9 Zellen wie in [52] 25 beschrieben, infiziert. Nach 3 Tagen in Kultur wurden die infizierten Zellen auf Bindung von TNF mittels ¹²⁵I-TNFα untersucht. Dazu wurden die transfektierten Zellen mit einer Pasteurpipette von der Zellkulturschale abgewaschen und bei einer Zelldichte von 5x10⁶ Zellen/ml Kulturmedium [52]. 30 das 10 ng/ml ¹²⁵I-TNF-α enthielt, sowohl in Anwesenheit wie Abwesenheit von 5 µg/ml nichtmarkiertem TNF-α resuspendiert und 2 Stunden auf Eis inkubiert. Danach wurden die Zellen mit reinem Kulturmedium gewaschen und die zellgebundene Radioaktivität in einem γ-Zähler gezählt (siehe 35 Tabelle 2).

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Tabelle 2

Zellen	Zellgebundene Radioaktivität pro 10^6 Zellen
5	
nichtinfizierte Zellen (Kontrolle)	60 cpm
infizierte Zellen	1600 \pm 330 cpm ¹⁾
10	¹⁾ Mittelwert und Standardabweichung aus 4 Experimenten

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Patentansprüche

1. Nichtlösliche Proteine und lösliche oder nicht-
lösliche Fragmente davon, die TNF binden, in homogener Form,
5 sowie deren physiologisch verträgliche Salze.

2. Verbindungen gemäss Anspruch 1, die durch Molekular-
gewichte gemäss SDS-PAGE unter nichtreduzierenden Be-
dingungen von etwa 55 kD und 75 kD charakterisiert sind.

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3. Verbindungen gemäss einem der Ansprüche 1 und 2, die
wenigstens eine der folgenden Aminosäuresequenzen enthalten:

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-
15 Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile;

Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys;

Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-
20 Gly-Ser-Thr-Cys;

Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu;

Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-
25 Glu-Lys-Pro-Leu;

Val-Phe-Cys-Thr;

Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-
30 Glu-Ala;

Leu-Cys-Ala-Pro;

Val-Pro-His-Leu-Pro-Ala-Asp;
35 Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro

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wobei X für einen nicht bestimmten Aminosäurerest steht.

5 4. Ein Verfahren zur Isolierung einer Verbindung gemäss einem der Ansprüche 1-3, dadurch gekennzeichnet, dass man im wesentlichen die folgenden Reinigungsschritte nacheinander ausführt: Herstellung eines Zellextraktes, Immunaффinitätschromatographie und/oder ein- oder mehrfache Ligandenaffinitätschromatographie, HPLC und präparative SDS-PAGE.

10 5. Pharmazeutische Präparate, dadurch gekennzeichnet, dass sie eine oder mehrere Verbindung(en) gemäss einem der Ansprüche 1-3, gewünschtenfalls in Kombination mit weiteren pharmazeutisch wirksamen Substanzen und/oder nicht-toxischen, inerten, therapeutisch verträglichen Trägermaterialien
15 enthalten.

20 6. Verwendung einer Verbindung gemäss einem der Ansprüche 1-3 zur Herstellung pharmazeutischer Präparate bzw. zur Behandlung von Krankheiten, bevorzugt solchen, bei denen TNF involviert ist.

7. Gegen eine Verbindung gemäss Ansprüche 1-3 gerichtete Antikörper.

25 8. DNA-Sequenzen, die für Proteine und lösliche oder nichtlösliche Fragmente davon, die TNF binden, kodieren.

30 9. Von DNA-Sequenzen gemäss Anspruch 8 kodierte rekombinante Proteine.

10. Vektoren, die DNA-Sequenzen gemäss Anspruch 8 enthalten und zur Expression der von diesen DNA-Sequenzen kodierten Proteinen in prokaryotischen- wie eukaryotischen Wirtssystemen geeignet sind.

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- 41 -

11. Prokaryotische- wie eukaryotische Wirtssysteme, die mit einem Vektor gemäss Anspruch 10 transformiert worden sind.

5 12. Ein Verfahren zur Herstellung von Verbindungen gemäss Anspruch 9, das dadurch gekennzeichnet ist, dass man ein wie in Anspruch 11 beanspruchtes transformiertes Wirtssystem in einem geeigneten Medium kultiviert und aus dem Wirtssystem selbst oder dem Medium solche Verbindungen
10 isoliert.

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-185 GAATTCGGGGGGGTTCAAGATCACTGGGACCAGGCCGTGATCTCTATGCCCGAGTCTCAA
-125 CCCTCAACTGTCACCCCAAGGCACTTGGGACGTCCTCGACAGACCGAGTCCCGGAAGCC
-65 CCAGCACTGCCGCTGCCACACTGCCCTGAGCCCAATGGGGGAGTGAGAGGCCATAGCTG
-28.
-30 MetGlyLeuSerThrValProAspLeuLeuProLeuValLeuLeuGluLeu
-5 TCTGGCATGGGCTCTCCACCGTGCCTGACCTGCTGCTGCCGCTGGTGCTCCTGGAGCTG
+1
-10 LeuValGlyIleTyrProSerGlyValIleGlyLeuValProHisLeuGlyAspArgGlu
55 TTGGTGGGAATATACCCCTCAGGGGTATTGGACTGGTCCCTCACCTAGGGGACAGGGAG

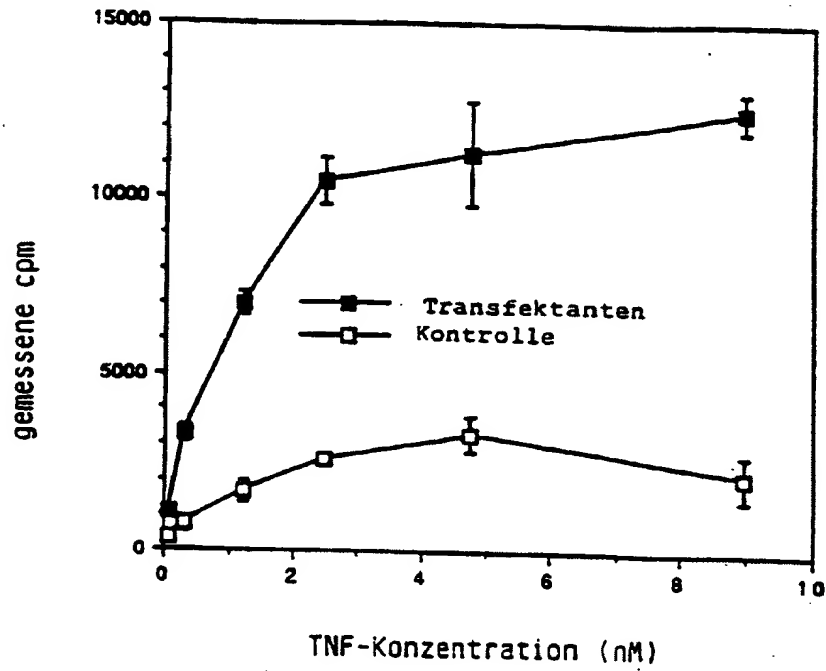
10 LysArgAspSerValCysProGlnGlyLysTyrIleHisProGlnAsnAsnSerIleCys
115 AAGAGAGATAGTGTGTGTCCCAAGGAAATATATCCACCCTCAAATAATTGATTGCT
30 CysThrLysCysHisLysGlyThrTyrLeuTyrAsnAspCysProGlyProGlyGlnAsp
175 TGTACCAAGTGCCACAAGGAACCTACTGTACAATGACTGTCCAGGCCCGGGGAGGAT
50 ThrAspCysArgGluCysGluSerGlySerPheThrAlaSerGluAsnHisLeuArgHis
235 ACGGACTGCAGGGAGTGTGAGAGCGGCTCCTTCACCGCTTCAGAAAACCACCTCAGACAC
70 CysLeuSerCysSerLysCysArgLysGluMetGlyGlnValGluIleSerSerCysThr
295 TGCCTCAGCTGCTCCAATGCCGAAAGGAAATGGGTGAGTGGAGATCTCTTCTGCACA
90 ValAspArgAspThrValCysGlyCysArgLysAsnGlnTyrArgHisTyrTrpSerGlu
355 GTGGACCGGGACACCGTGTGTGGTGCAGGAAGAACCAGTACCGGCATTATTGGAGTGAA

110 AsnLeuPheGlnCysPheAsnCysSerLeuCysLeuAsnGlyThrValHisLeuSerCys
415 AACCTTTTCCAGTGCTTCAATTGCAGCCTCTGCCTCAATGGGACCGTGCACCTCTCTCTGC
130 GlnGluLysGlnAsnThrValCysThrCysHisAlaGlyPhePheLeuArgGluAsnGlu
475 CAGGAGAAACAGAACACCGTGTGCACCTGCCATGCAGGTTTCTTTCTAAGAGAAAACGAG
150 CysValSerCysSerAsnCysLysLysSerLeuGluCysThrLysLeuCysLeuProGln
535 TGTGTCTCCTGTAGTAAGTGTAAAGAAAGCCTGGAGTGCACGAAGTTGTGCCTACCCAG
170 IleGluAsnValLysGlyThrGluAspSerGlyThrThrValLeuLeuProLeuValIle
595 ATTGAGAATGTTAAGGGCACTGAGGACTCAGGCACCACAGTGGTGTGCCCCCTGGTCATT
190 PhePheGlyLeuCysLeuLeuSerLeuLeuPheIleGlyLeuMetTyrArgTyrGlnArg
655 TTCTTTGGTCTTTGCCTTTTATCCCTCCTCTTCATTGGTTAATGTATCGCTACCAACGG
210 TrpLysSerLysLeuTyrSerIleValCysGlyLysSerThrProGluLysGluGlyGlu
715 TGGAAGTCCAAGCTCTACTCCATTGTTGTGGGAAATCGACACCTGAAAAGAGGGGGAG

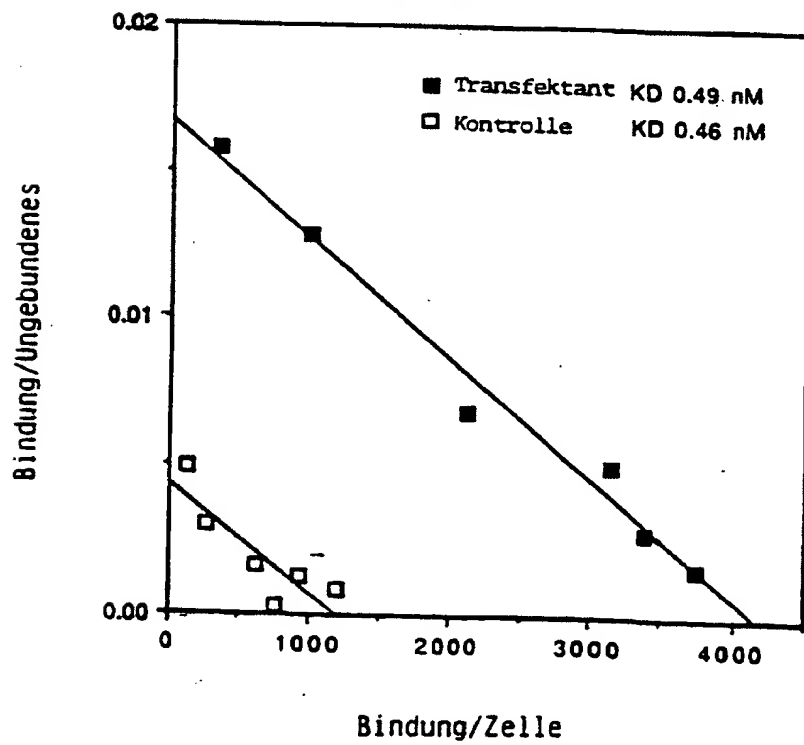
230 LeuGluGlyThrThrThrLysProLeuAlaProAsnProSerPheSerProThrProGly
775 CTTGAAGGAACCTACTACTAAGCCCTGGCCCAACCAAGCTTCAGTCCCACTCCAGGC
250 PheThrProThrLeuGlyPheSerProValProSerSerThrPheThrSerSerSerThr
835 TTCACCCCAACCTGGGCTTCAGTCCCGTGGCCAGTTCCACCTTCACCTCCAGCTCCACC
270 TyrThrProGlyAspCysProAsnPheAlaAlaProArgArgGluValAlaProProTyr
895 TATACCCCGGTGACTGTCCCAACTTTGGCGCTCCCCGCAGAGAGGTGCCACCACCCTAT
290 GlnGlyAlaAspProIleLeuAlaThrAlaLeuAlaSerAspProIleProAsnProLeu
955 CAGGGGGCTGACCCCATCCTTGCGACAGCCCTCGCCTCCGACCCCATCCCCAACCCCTT

310 GlnLysTrpGluAspSerAlaHisLysProGlnSerLeuAspThrAspAspProAlaThr
1015 CAGAAGTGGGAGGACAGCGCCCAAGCCACAGAGCCTAGACACTGATGACCCCGCGACG
330 LeuTyrAlaValValGluAsnValProProLeuArgTrpLysGluPheValArgArgLeu
1075 CTGTACGCCGTGGTGGAGAACGTGCCCGCTTGGCTGGAAGGAATTCGTGCGGCGCCTA
350 GlyLeuSerAspHisGluIleAspArgLeuGluLeuGlnAsnGlyArgCysLeuArgGlu
1135 GGGCTGAGCGACCAAGATCGATCGGCTGGAGCTGCAGAACGGGCGCTGCCTGCGCGAG
370 AlaGlnTyrSerMetLeuAlaThrTrpArgArgArgThrProArgArgGluAlaThrLeu
1195 GCGCAATACAGCATGCTGGCGACCTGGAGGCGGCGCACGCCGCGGCGCGAGGCCACGCTG
390 GluLeuLeuGlyArgValLeuArgAspMetAspLeuLeuGlyCysLeuGluAspIleGlu
1255 GAGCTGCTGGGACCGCTGCTCCGCGACATGGACCTGCTGGGCTGCCTGGAGGACATCGAG
410 GluAlaLeuCysGlyProAlaAlaLeuProProAlaProSerLeuLeuArg
1315 GAGGCGCTTTGCGGCCCCGCGCCCTCCCGCCCGCGCCAGTCTTCTCAGATGAGGCTGC
1375 GCGGCTGCGGGCAGCTCTAAGGACCGTCTGCGAGATCGCCTTCCAACCCCACTTTTTTC
1435 TGGAAAGGAGGGGTCTGCGAGGGCAAGCAGGAGCTAGCAGCCGCGCTACTTGGTGCTAAC
1495 CCTCGATGTACATAGCTTTTCTCAGCTGCCGCGCGCGCGACAGTCAGCCCTGTGCG
1555 CCGGAGAGAGGTGCGCGTGGGCTCAAGAGCCTGAGTGGGTGGTTTGGGAGGATGAGGG
1615 ACGCTATGCCTCATGCCGTTTGGGTGTCTCACCAGCAAGGCTGCTCGGGGCCCCCTG
1675 GTTCGTCCCTGAGCCTTTTACAGTGCATAAGCAGTTTTTTTGTGTTTTGTTTTGTTTT
1735 GTTTTGTGTTTTAAATCAATCATGTTACACTAATAGAACTTGGCACTCCTGTGCCCTCTG
1795 CCTGGACAAGCACATAGCAAGCTGAAGTGTCTAAGGCAGGGGCGAGCACGGAACAATGG
1855 GGCCTTCAGCTGGAGCTGTGGACTTTTGTACATACACTAAAATTCTGAAGTTAAAAAAA
1915 AACCCGAATTC

Figur 2A



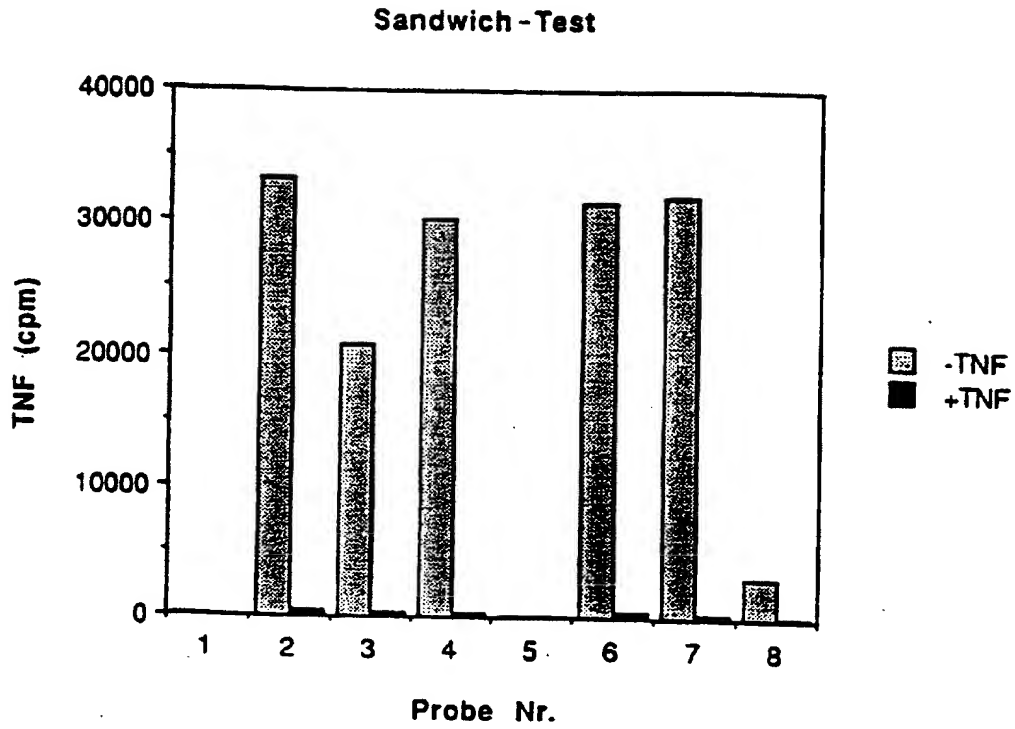
Figur 2B



Exemplar
Exemplare immutabile

M 31.08.90

Figur 3



Figur 4

1 SerAspSerValCysAspSerCysGluAspSerThrTyrThrGlnLeuTrpAsnTrpVal
1 TCGGACTCCGTGTGTGACTCCTGTGAGGACAGCACATACACCCAGCTCTGGAAGTGGGT

21 ProGluCysLeuSerCysGlySerArgCysSerSerAspGlnValGluThrGlnAlaCys
61 CCCGAGTGCTTGAGCTGTGGCTCCCGCTGTAGCTCTGACCAGGTGGAACTCAAGCCTGC

41 ThrArgGluGlnAsnArgIleCysThrCysArgProGlyTrpTyrCysAlaLeuSerLys
121 ACTCGGGAACAGAACCGCATCTGCACCTGCAGGCCCGGCTGGTACTGCGCGCTGAGCAG

61 GlnGluGlyCysArgLeuCysAlaProLeuProLysCysArgProGlyPheGlyValAla
181 CAGGAGGGGTGCCGCTGTGCGCGCCGCTGCCGAAGTGCCGCCCGGGCTTCGGCGTGGCC

81 ArgProGlyThrGluThrSerAspValValCysLysProCysAlaProGlyThrPheSer
241 AGACCAGGAAGTGAACATCAGACGTGGTGTGCAAGCCCTGTGCCCCGGGGACGTTCTCC

101 AsnThrThrSerSerThrAspIleCysArgProHisGlnIleCysAsnValValAlaIle
301 AACACGACTTCATCCACGGATATTTGCAGGCCCCACCAGATCTGTAACTGGTGGCCATC

121 ProGlyAsnAlaSerArgAspAlaValCysThrSerThrSerProThrArgSerMetAla
361 CCTGGGAATGCAGCAGGGATGCAGTCTGCACGTCCACGTCCCCACCCGGAGTATGGCC

141 ProGlyAlaValHisLeuProGlnProValSerThrArgSerGlnHisThrGlnProSer
421 CCAGGGGCAGTACACTTACCCAGCCAGTGTCCACACGATCCCAACACACGCAGCCAGT

161 ProGluProSerThrAlaProSerThrSerPheLeuLeuProMetGlyProSerProPro
481 CCAGAACCCAGCACTGCTCCAGCACCTCCTTCTGCTCCCAATGGGCCCCAGCCCCCA

181 AlaGluGlySerThrGlyAspPheAlaLeuProValGlyLeuIleValGlyValThrAla
541 GCTGAAGGGAGCACTGGCGACTTCGCTCTTCCAGTTGGACTGATTGTGGGTGTGACAGCC

201 LeuGlyLeuLeuIleIleGlyValValAsnCysValIleMetThrGlnValLysLysLys
601 TTGGGTCTACTAATAATAGGAGTGGTGAAGTGTGTCATCATGACCCAGGTGAAAAAGAG

221 ProLeuCysLeuGlnArgGluAlaLysValProHisLeuProAlaAspLysAlaArgGly
661 CCCTTGTCCTGCAGAGAGAGCCAGGTGCCTCACTTGCTGCTGCGATAAGGCCCGGGT

241 ThrGlnGlyProGluGlnGlnHisLeuLeuIleThrAlaProSerSerSerSerSerSer
721 ACACAGGGCCCCGAGCAGCAGCACCTGCTGATCACAGCGCCGAGCTCCAGCAGCAGCTCC

261 LeuGluSerSerAlaSerAlaLeuAspArgArgAlaProThrArgAsnGlnProGlnAla
781 CTGGAGAGCTCGGCCAGTGCGTTGGACAGAGGGGCGCCCACTCGGAACAGCCACAGGCA

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Figur 4. (Fortsetzung)

281 ProGlyValGluAlaSerGlyAlaGlyGluAlaArgAlaSerThrGlySerSerAlaAsp
841 CCAGGCGTGGAGGCCAGTGGGGCCGGGGAGGCCCGGGCCAGCACCGGGAGCTCAGCAGAT
301 SerSerProGlyGlyHisGlyThrGlnValAsnValThrCysIleValAsnValCysSer
901 TCTTCCCTGGTGGCCATGGGACCCAGGTCAATGTCACCTGCATCGTGACGTCTGTAGC
321 SerSerAspHisSerSerGlnCysSerSerGlnAlaSerSerThrMetGlyAspThrAsp
961 AGCTCTGACCACAGCTCACAGTGCTCCTCCAGGCCAGCTCCACATGGGAGACACAGAT
341 SerSerProSerGluSerProLysAspGluGlnValProPheSerLysGluGluCysAla
1021 TCCAGCCCCCTCGGAGTCCCCGAGGACGAGCAGGTCCCCCTCTCCAGGAGGATGTGCC
361 PheArgSerGlnLeuGluThrProGluThrLeuLeuGlySerThrGluGluLysProLeu
1081 TTTCGGTCACAGCTGGAGACGCCAGAGACCCTGCTGGGGAGCACCGAGAGAGCCCCCTG
381 ProLeuGlyValProAspAlaGlyMetLysProSer
1141 CCCCTTGGAGTGCCTGATGCTGGGATGAAGCCAGTTAACCAGGCCGGTGTGGGCTGTGT
1201 CGTAGCCAGGTGGCTGAGCCCTGGCAGGATGACCCTGCGAAGGGGCCCTGGTCTTCCA
1261 GGCCCCCACCCTAGGACTCTGAGGCTCTTTCTGGGCCAAGTTCTCTAGTGCCCTCCAC
1321 AGCCGCAGCCTCCCTCTGACCTGCAGGCCAGAGCAGAGGCAGCGAGTTGTGGAAGCCT
1381 CTGCTGCCATGGCGTGCCCTCTCGGAGGCTGGCTGGGCATGGACGTTCTGGGGCATGCT
1441 GGGGCAAGTCCCTGAGTCTCTGTGACCTGCCCGCCAGCTGCACCTGCCAGCCTGGCTT
1501 CTGGAGCCCTTGGGTTTTTGTGTTGTTGTTGTTGTTGTTGTTGTTTCTCCCCCTGGGC
1561 TCTGCCAGCTCTGGCTTCCAGAAACCCAGCATCCTTTTCTGCAGAGGGGCTTTCTGG
1621 AGAGGAGGGATGCTGCCTGAGTCACCCATGAAGACAGGACAGTGCTTCAGCCTGAGGCTG
1681 AGACTGCGGGATGGTCCTGGGGCTCTGTGCAGGGAGGAGGTGGCAGCCCTGTAGGGACG
1741 GGGTCCTTCAAGTTAGCTCAGGAGGCTTGGAAAGCATCACCTCAGGCCAGGTGCAGTGGC
1801 TCACGCCTATGATCCCAGCACTTTGGGAGGCTGAGGCGGGTGGATCACCTGAGGTTAGGA
1861 GTTCGAGACCAGCCTGGCCAACATGGTAAACCCCATCTCTACTAAAAATACAGAAATTA
1921 GCCGGGCGTGGTGGCGGGCACCTATAGTCCCAGCTACTCAGAGCCTGAGGCTGGGAAT
1981 CGTTTGAACCCGGGAGCGGAGGTTCAGGGAGCCGAGATCAGCCACTGCACTCCAGCC
2041 TGGGCGACAGAGCGAGAGTCTGTCTCAAAGAAAAAAGACCCGCTCCAATGCT
2101 AACTTGTCTTTTGTACCATGGTGTGAAGTCAGATGCCAGAGGGGCCAGGCAGGCCAC
2161 CATATTCAAGTGCTGTGGCCTGGGCAAGATAACGCACTTCTACTAGAAATCTGCCAATTT
2221 TTTAAAAAGTAAGTACCACTCAGGCCAACAGCCAACGACAAAGCCAACCTCTGCCAGC
2281 CACATCCAAACCCCCACCTGCCATTTGCACCTCCGCTTCACTCCGGTGTGCCTGCAG



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Munich,
Munich, le

29/11/06

Präsident des Europäischen Patentamts:
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For the President of the European Patent Office.

Le Président de l'Office européen des brevets
p.o.

[Signature]
DM Ernst Erdmann

(19)



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(22) Anmeldetag: 31.08.1990

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TNF-binding proteins

Protéines qui lient le TNF

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Anmerkung: Innerhalb von neun Monaten nach der Bekanntmachung des Hinweises auf die Erteilung des europäischen Patents kann jedermann beim Europäischen Patentamt gegen das erteilte europäische Patent Einspruch einlegen. Der Einspruch ist schriftlich einzureichen und zu begründen. Er gilt erst als eingelegt, wenn die Einspruchsgebühr entrichtet worden ist. (Art. 99(1) Europäisches Patentübereinkommen).

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Bemerkungen:

Die Akte enthält technische Angaben, die nach dem Eingang der Anmeldung eingereicht wurden und die nicht in dieser Patentschrift enthalten sind.

Beschreibung

[0001] Tumor Nekrosis Faktor α (TNF α , auch Cachectin), auf Grund seiner haemorrhagisch-nekrotisierenden Wirkung auf bestimmte Tumoren entdeckt, und Lymphotoxin (TNF β) sind zwei nahe verwandte Peptidfaktoren [3] aus der Klasse der Lymphokine/Cytokine, die im folgenden beide als TNF bezeichnet werden [siehe Uebersichtsarbeiten 2 und 3]. TNF verfügt über ein breites zelluläres Wirkungsspektrum. Beispielsweise besitzt TNF inhibierende oder cytotoxische Wirkung auf eine Reihe von Tumorzelllinien [2,3], stimuliert die Proliferation von Fibroblasten und die phagozytierende/cytotoxische Aktivität von myeloischen Zellen [4,5,6], induziert Adhäsionsmoleküle in Endothelzellen oder übt eine inhibierende Wirkung auf Endothel aus [7,8,9,10], inhibiert die Synthese von spezifischen Enzymen in Adipozyten [11] und induziert die Expression von Histokompatibilitätsantigenen [12]. Manche dieser TNF-Wirkungen werden über eine Induktion von anderen Faktoren oder durch synergistische Effekte mit anderen Faktoren, wie beispielsweise Interferonen oder Interleukinen erzielt [13-16].

[0002] TNF ist bei einer Reihe von Pathologischen Zuständen, beispielsweise Schockzuständen bei Meningococcus-Sepsis [17], bei der Entwicklung von Autoimmun-Glomerulonephritis bei Mäusen [18] oder bei cerebraler Malaria bei Mäusen [19] und beim Menschen [41] involviert. Ganz allgemein scheinen die toxischen Wirkungen von Endotoxin durch TNF vermittelt zu sein [20]. Weiterhin kann TNF wie Interleukin-1 Fieber auslösen [39]. Auf Grund der pleiotropen funktionellen Eigenschaften von TNF kann man annehmen, dass TNF in Wechselwirkung mit anderen Cytokinen bei einer ganzen Reihe weiterer pathologischer Zustände als Mediator von Immunantwort, Entzündung oder anderen Prozessen beteiligt ist.

[0003] Diese biologischen Effekte werden durch TNF über spezifische Rezeptoren vermittelt, wobei nach heutigem Wissensstand sowohl TNF α wie TNF β an die gleichen Rezeptoren binden [21]. Verschiedene Zelltypen unterscheiden sich in der Anzahl von TNF-Rezeptoren [22,23,24]. Solche ganz allgemein gesprochen TNF-bindenden Proteine (TNF-BP) wurden durch kovalente Bindung an radioaktiv markiertes TNF nachgewiesen [24-29], wobei die folgenden scheinbaren Molekulargewichte der erhaltenen TNF/TNF-BP-Komplexe ermittelt wurden: 95/100 kD und 75 kD [24], 95 kD und 75 kD [25], 138 kD, 90 kD, 75 kD und 54 kD [26], 100 \pm 5 kD [27], 97 kD und 70 kD [28] und 145 kD [29]. Mittels anti-TNF-Antikörper-Immunoaffinitätschromatographie und präparativer SDS-Polyacrylamidgelelektrophorese (SDS-PAGE) konnte ein solcher TNF/TNF-BP-Komplex isoliert werden [27]. Die reduktive Spaltung dieses Komplexes und anschließende SDS-PAGE-Analyse ergab mehrere Banden, die allerdings nicht auf TNF-Bindeaktivität getestet wurden. Da die spezifischen Bedingungen, die zu der Spaltung des Komplexes verwendet werden müssen, zur Inaktivierung des Bindeproteins führen [31], ist letzteres auch nicht möglich gewesen. Die Anreicherung von löslichen TNF-BP aus dem humanen Serum oder Urin mittels Ionenaustauscher-Chromatographie und Gelfiltration (Molekulargewichte im Bereich von 50 kD) wurde von Olsson et al. beschrieben [30].

[0004] Brockhaus et al. [32] erhielten durch TNF α -Ligandenaffinitätschromatographie und HPLC aus Membranextrakten von HL60-Zellen eine angereicherte TNF-BP-Präparation, die wiederum als Antigenpräparation zur Herstellung von monoklonalen Antikörpern gegen TNF-BP verwendet wurde. Unter Verwendung eines solchen immobilisierten Antikörpers (Immunoaffinitätschromatographie) wurde mittels TNF α -Ligandenaffinitätschromatographie und HPLC von Loetscher und Brockhaus [31] aus einem Extrakt von humaner Placenta eine angereicherte Präparation von TNF-BP erhalten, die in der SDS-PAGE-Analyse eine starke breite Bande bei 35 kD, eine schwache Bande bei etwa 40 kD und eine sehr schwache Bande im Bereich zwischen 55 kD und 60 kD ergab. Im übrigen zeigte das Gel im Bereich von 33 kD bis 40 kD einen Proteinintergrundschmier. Die Bedeutung der so erhaltenen Proteinbanden war jedoch im Hinblick auf die Heterogenität des verwendeten Ausgangsmaterials (Placenta-Gewebe; vereinigtes Material aus mehreren Placenten) nicht klar.

[0005] Fusionsproteine bestehend aus der extrazellulären Domäne von nicht zur Immunoglobulinfamilie gehörenden membranständigen humanen Proteinen, wie beispielsweise Tumor-Nekrose-Faktor-Rezeptor, und Immunoglobulinen, insbesondere der konstante Teil der schweren Kette, wobei die Fusion bevorzugt an den Hinge Bereich erfolgt, sind in EP 464533 erwähnt. Die Herstellung der erfindungsgemässen p55-Tumor-Nekrose-Faktor-Rezeptor-Fusionsproteine ist allerdings weder in EP 464533 offenbar noch sind solche Fusionsproteine und deren vorteilhaften Eigenschaften überhaupt dort genannt.

[0006] Die vorliegende Erfindung betrifft somit weiterhin DNA-Sequenzen, die eine Kombination aus zwei Teil-DNA-Sequenzen umfassen, wobei die eine Teilsequenz für löslichen TNF-bindende Fragmente von TNF-Rezeptoren kodiert und wobei solche DNA-Sequenzen aus den folgenden auswählbar sind:

(a) Fragmente von DNA-Sequenzen, wie sie in Figur 1 dargestellt sind, wie deren komplementäre Stränge;

(b) DNA-Sequenzen, die mit wie unter (a) definierten Fragmenten hybridisieren;

(c) DNA-Sequenzen, die auf Grund der Entartung des genetischen Codes nicht mit Sequenzen, wie unter (a) und (b) definiert, hybridisieren, aber die für Polypeptide mit genau gleicher Aminosäuresequenz kodieren;

und die andere Teil-Sequenz, für alle Domänen ausser der ersten Domäne der konstanten Region der schweren Kette von humanen Immunglobulinen der Klasse IgG kodiert. Bevorzugt sind solche DNA-Sequenzen, welche von Nukleotid -185 bis 633 bzw. von Nukleotid -14 bis 633 der in Abbildung 1 gezeigten Sequenz reichen.

[0007] Die vorliegende Erfindung betrifft natürlich auch die von solchen DNA-Sequenzen kodierten rekombinanten Proteine. Selbstverständlich sind dabei auch solche Proteine umfasst, in deren Aminosäuresequenzen, beispielsweise mittels gezielter Mutagenese, Aminosäuren so ausgetauscht worden sind, dass dadurch die Aktivität der TNF-BP-Fragmente, nämlich die Bindung von TNF oder die Wechselwirkung mit anderen, an der Signalübertragung beteiligten Membrankomponenten, in einer gewünschten Art verändert oder erhalten wurden. Aminosäureaustausche in Proteinen und Peptiden, die im allgemeinen die Aktivität solcher Moleküle nicht verändern, sind im Stand der Technik bekannt und beispielsweise von H. Neurath und R.L. Hill in "The Proteins" (Academic Press, New York, 1979, siehe besonders Figur 6, Seite 14) beschrieben. Die am häufigsten vorkommenden Austausche sind: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly, sowie solche in umgekehrter Weise. Die vorliegende Erfindung betrifft ferner Vektoren, die erfindungsgemässe DNA-Sequenzen enthalten und zur Transformation von geeigneten pro- wie eukaryotischen Wirtssystemen geeignet sind und deren Verwendung zur Expression der von den erfindungsgemässen DNA-Sequenzen kodierten Proteine führt. Schliesslich betrifft die vorliegende Erfindung auch noch mit solchen Vektoren transformierte Pro- wie eukaryotische Wirtssysteme, wie Verfahren zur Herstellung von erfindungsgemässen rekombinanten Verbindungen durch Kultivierung solcher Wirtssysteme und anschliessende Isolierung dieser Verbindungen aus den Wirtssystemen selbst oder deren Kulturüberständen.

[0008] Gegenstand der vorliegenden Erfindung sind auch pharmazeutische Präparate, die wenigstens eines der erfindungsgemässen Proteine, gewünschtenfalls in Verbindung mit weiteren pharmazeutisch wirksamen Substanzen und/oder nicht-toxischen, inerten, therapeutisch verträglichen Trägermaterialien enthalten.

[0009] Die vorliegende Erfindung betrifft schliesslich die Verwendung solcher erfindungsgemässen Proteine einerseits zur Herstellung pharmazeutischer Präparate bzw. andererseits zur Behandlung von Krankheiten, bevorzugt solchen, in deren Verlauf TNF involviert ist.

[0010] Ausgangsmaterial für TNF-BP sind ganz allgemein Zellen, die solche TNF-BP in membrangebundener Form enthalten und die dem Fachmann ohne Beschränkungen allgemein zugänglich sind, wie beispielsweise HL60- [ATCC Nr. CCL 240], U 937- [ATCC Nr. CRL 1593], SW 480- [ATCC Nr. CCL 228] und HEP2-Zellen [ATCC Nr. CCL 23]. Diese Zellen können nach bekannten Methoden des Standes der Technik [40] oder zum Erzielen hoher Zelldichten nach dem bereits allgemein und im Detail für HL60-Zellen in Beispiel 2 beschriebenen Verfahren kultiviert werden. TNF-BP können dann nach bekannten Methoden des Standes der Technik mittels geeigneter Detergenzien, beispielsweise Triton X-114, 1-O-n-Octyl- β -D-glucopyranosid (Octylglucosid), oder 3-[(3-Cholylamidopropyl)-dimethylammonio]-1-propan sulfonat (CHAPS), im besonderen mittels Triton X-100, aus den aus dem Medium abzentrifugierten und gewaschenen Zellen extrahiert werden. Zum Nachweis solcher TNF-BP können die üblicherweise verwendeten Nachweismethoden für TNF-BP, beispielsweise eine Polyäthylenglykol-induzierte Fällung des 125 I-TNF/TNF-BP-Komplexes [27], im besonderen Filterbindungstests mit radioaktiv markiertem TNF gemäss Beispiel 1, verwendet werden. Zur Gewinnung der TNF-BP können die generell zur Reinigung von Proteinen, insbesondere von Membranproteinen, verwendeten Methoden des Standes der Technik, wie beispielsweise Ionenaustausch-Chromatographie, Gelfiltration, Affinitätschromatographie, HPLC und SDS-PAGE verwendet werden. Besonders bevorzugte Methoden zur Herstellung von TNF-BP sind Affinitätschromatographie, insbesondere mit TNF- α als an die Festphase gebundenen Liganden und Immunaaffinitätschromatographie, HPLC und SDS-PAGE. Die Elution von mittels SDS-PAGE aufgetrennten TNF-BP Banden kann nach bekannten Methoden der Proteinchemie erfolgen, beispielsweise mittels Elektroelution nach Hunkapiller et al. [34], wobei nach heutigem Stand des Wissens die dort angegebenen Elektro-Dialysezeiten generell zu verdoppeln sind. Danach noch verbleibende Spuren von SDS können dann gemäss Bosserhoff et al. [50] entfernt werden.

[0011] Die so gereinigten TNF-BP können mittels der im Stand der Technik bekannten Methoden der Peptidchemie, wie beispielsweise N-terminale Aminosäuresequenzierung oder enzymatische wie chemische Peptidspaltung charakterisiert werden. Durch enzymatische oder chemische Spaltung erhaltene Fragmente können nach gängigen Methoden, wie beispielsweise HPLC, aufgetrennt und selbst wieder N-terminal sequenziert werden. Solche Fragmente, die selbst noch TNF binden, können mittels der obengenannten Nachweismethoden für TNF-BP identifiziert werden.

[0012] Ausgehend von der so erhältlichen Aminosäuresequenzinformation oder den in Figur 1 dargestellten DNA- wie Aminosäuresequenzen können unter Beachtung der Degeneration des genetischen Codes nach im Stand der Technik bekannten Methoden geeignete Oligonukleotide hergestellt werden [51]. Mittels dieser können dann wiederum nach bekannten Methoden der Molekularbiologie [42,43] cDNA- oder genomische DNA-Banken nach Klonen, die für TNF-BP kodierende Nukleinsäuresequenzen enthalten, abgesucht werden. Ausserdem können mittels der Polymerase-Kettenreaktion (PCR) [49] cDNA-Fragmente kloniert werden, indem von zwei auseinanderliegenden, relativ kurzen Abschnitten der Aminosäuresequenz unter Beachtung des genetischen Codes vollständig degenerierte und in ihrer

Komplementarität geeignete Oligonucleotide als "Primer" eingesetzt werden, wodurch das zwischen diesen beiden Sequenzen liegende Fragment amplifiziert und identifiziert werden kann. Die Bestimmung der Nukleotidsequenz eines derartigen Fragmentes ermöglicht eine unabhängige Bestimmung der Aminosäure-Sequenz des Proteinfragments, für das es kodiert. Die mittels der PCR erhältlichen cDNA-Fragmente können ebenfalls, wie bereits für die Oligonukleotide selbst beschrieben, nach bekannten Methoden zum Aufsuchen von für TNF-BP kodierende Nukleinsäuresequenzen enthaltenden Klonen aus cDNA- bzw. genomische DNA-Banken verwendet werden. Solche Nukleinsäuresequenzen können dann nach bekannten Methoden sequenziert werden [42]. Aufgrund der so bestimmten wie der für bestimmte Rezeptoren bereits bekannten Sequenzen können solche Teilsequenzen, die für lösliche TNF-BP-Fragmente kodieren, bestimmt und mittels bekannter Methoden aus der Gesamtsequenz herausgeschnitten werden [42].

[0013] Die gesamte Sequenz oder solche Teilsequenzen können dann mittels bekannter Methoden in im Stand der Technik beschriebene Vektoren zu deren Vervielfältigung wie Expression in Prokaryoten integriert werden [42]. Geeignete Prokaryotische Wirtsorganismen stellen beispielsweise gram-negative wie gram-Positive Bakterien, wie beispielsweise E. coli Stämme, wie E. coli HB 101 [ATCC Nr. 33 694] oder E. coli W3110 [ATCC Nr. 27 325] oder B. subtilis Stämme dar.

[0014] Weiterhin können erfindungsgemäße Nukleinsäuresequenzen in geeignete Vektoren zur Vermehrung wie Expression in eukaryotischen Wirtszellen, wie beispielsweise Hefe, Insekten- und Säugerzellen, mittels bekannter Methoden integriert werden. Expression solcher Sequenzen erfolgt bevorzugt in Säuger- wie Insektenzellen.

[0015] Ein typischer Expressionsvektor für Säugerzellen enthält ein effizientes Promotorelement, um eine gute Transkriptionsrate zu erzielen, die zu exprimierende DNA-Sequenz und Signale für eine effiziente Termination und Polyadenylierung des Transkripts. Weitere Elemente, die verwendet werden können, sind "Enhancer", welche zu nochmals verstärkter Transkription führen und Sequenzen, welche z.B. eine längere Halbwertszeit der mRNA bewirken können. Zur Expression von Nukleinsäuresequenzen, denen das endogene für ein Signalpeptid kodierende Sequenzstück fehlt, können Vektoren verwendet werden, die solche geeignete Sequenzen, die für Signalpeptide von anderen bekannten Proteinen kodieren, enthalten. Siehe beispielsweise der von Cullen, B.R. in Cell 46, 973-982 (1986) beschriebene Vektor pLJ268 oder auch bei Sharma, S. et al. in "Current Communications in Molecular Biology", ed. by Gething, M.J., Cold Spring Harbor Lab. (1985), Seiten 73-78.

[0016] Die meisten Vektoren, die für eine transiente Expression einer bestimmten DNA-Sequenz in Säugerzellen verwendet werden, enthalten den Replikationsursprung des SV40 Virus. In Zellen, die das T-Antigen des Virus exprimieren, (z.B. COS-Zellen), werden diese Vektoren stark vermehrt. Eine vorübergehende Expression ist aber nicht auf COS-Zellen beschränkt. Im Prinzip kann jede transfektierbare Säugerzelllinie hierfür verwendet werden. Signale, die eine starke Transkription bewirken können, sind z.B. die frühen und späten Promotoren von SV40, der Promoter and Enhancer des "major immediate-early" Gens des HCMV (humaner Cytomegalovirus), die LTRs ("long terminal repeats") von Retroviren, wie beispielsweise RSV, HIV und MMTV. Es können aber auch Signale von zellulären Genen, wie z.B. die Promotoren des Aktin- und Collagenase-Gens, verwendet werden.

[0017] Alternativ können aber auch stabile Zelllinien, die die spezifische DNA-Sequenz im Genom (Chromosom) integriert haben, erhalten werden. Hierzu wird die DNA-Sequenz zusammen mit einem selektierbaren Marker, z.B. Neomycin, Hygromycin, Dihydrofolat-Reduktase (dhfr) oder Hypoxanthin-Guanin-Phosphoribosyltransferase (hgpt) kotransfiziert. Die stabil ins Chromosom eingebaute DNA-Sequenz kann auch noch stark vermehrt werden. Ein geeigneter Selektionsmarker hierfür ist beispielsweise die Dihydrofolat-Reduktase (dhfr). Säugerzellen (z.B. CHO-Zellen), welche kein intaktes dhfr-Gen enthalten, werden hierbei nach erfolgter Transfektion mit steigenden Mengen von Methotrexat inkubiert. Auf diese Weise können Zelllinien erhalten werden, welche mehr als tausend Kopien der gewünschten DNA-Sequenz enthalten.

[0018] Säugerzellen, welche für die Expression verwendet werden können, sind z.B. Zellen der menschlichen Zelllinien Hela [ATCC CCL2] und 293 [ATCC CRL 1573], sowie 3T3- [ATCC CCL 163] und L-Zellen, z.B. [ATCC CCL 149], (CHO)-Zellen [ATCC CCL 61], BHK [ATCC CCL 10]-Zellen sowie die CV 1 [ATCC CCL 70]- und die COS-Zelllinien [ATCC CRL 1650, CRL 1651].

[0019] Geeignete Expressionsvektoren umfassen beispielsweise Vektoren wie pBC12MI [ATCC 67 109], pSV2dhfr [ATCC 37 146], pSVL [Pharmacia, Uppsala, Sweden], pRSVCat [ATCC 37 152] und pMSG [Pharmacia, Uppsala, Sweden]. Besonders bevorzugte Vektoren sind die in Beispiel 9 verwendeten Vektoren "pK19" und "pN123". Diese können aus den mit ihnen transformierten E. coli-Stämmen HB101(pK19) und HB101(pN123) nach bekannten Methoden isoliert werden [42]. Diese E. coli-Stämme wurden am 26. Januar 1990 bei der Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, BRD unter DSM 5761 für HB101(pK19) und DSM 5764 für HB101(pN123) hinterlegt. Zur Expression der erfindungsgemäßen Proteine, eignen sich besonders pSV2 abgeleitete Vektoren wie beispielsweise von German, C. in "DNA Cloning" [Vol. II., ed. von Glover, D.M., IRL Press, Oxford, 1985] beschrieben. Besonders bevorzugte Vektoren sind die bei der Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, BRD hinterlegten und in der Europäischen Patentanmeldung Nr. 90107393.2 genau beschriebenen Vektoren pCD4-H_μ (DSM 5315), pCD4-H_γ1 (DSM 5314) und pCD4-H_γ3 (DSM 5523). Besagte Europäische Patentschrift wie die in Beispiel 11 angegebenen äquivalenten Anmeldungen enthalten auch Angaben

bezüglich der weiteren Verwendung dieser Vektoren zur Expression von chimären Proteinen (siehe auch Beispiel 11) wie zur Konstruktion von Vektoren für die Expression von solchen chimären Proteinen mit anderen Immunglobulinanteilen.

[0020] Die Art und Weise wie die Zellen transfektiert werden hängt vom gewählten Expressionssystem und Vektorsystem ab. Eine Uebersicht über diese Methoden findet man z.B. bei Pollard et al., "DNA Transformation of Mammalian Cells" in "Methods in Molecular Biology" [Nucleic Acids Vol. 2, 1984, Walker, J.M., ed, Humana, Clifton, New Jersey]. Weitere Methoden findet man bei Chen und Okayama ["High-Efficiency Transformation of Mammalian Cells by Plasmid DNA", Molecular and Cell Biology 7, 2745-2752, 1987] und bei Felgner [Felgner et al., "Lipofectin: A highly efficient, lipid-mediated DNA-transfection procedure", Proc. Nat. Acad. Sci. USA 84, 7413-7417, 1987].

[0021] Zur Expression in Insektenzellen kann das Baculovirus-Expressions-System, welches schon für die Expression einer Reihe von Proteinen erfolgreich eingesetzt worden ist (für eine Uebersicht siehe Luckow and Summers, Bio/Technology 6, 47-55, 1988), verwendet werden. Rekombinante Proteine können authentisch oder als Fusionsproteine hergestellt werden. Die so hergestellten Proteine können auch modifiziert, wie beispielsweise glykosyliert (Smith et al., Proc. Nat. Acad. Sci. USA 82, 8404-8408, 1987) sein. Für die Herstellung eines rekombinanten Baculovirus, der das gewünschte Protein exprimiert, verwendet man einen sogenannten "Transfervektor". Hierunter versteht man ein Plasmid, welches die heterologe DNA-Sequenz unter der Kontrolle eines starken Promoters, z.B. dem des Polyhedrinogens, enthält, wobei diese auf beiden Seiten von viralen Sequenzen umgeben ist. Besonders bevorzugte Vektoren sind die in Beispiel 10 verwendeten Vektoren "pN113", "pN119" und "pN124". Diese können aus den mit ihnen transformierten E. coli-Stämmen HB101(pN113), HB101(pN119) und HB101(pN124) nach bekannten Methoden isoliert werden [42]. Diese E. coli-Stämme wurden am 26. Januar 1990 bei der Deutschen Sammlung von Mikroorganismen und Zellkulturen-GmbH (DSMZ) in Braunschweig, BRD, unter DSM 5762 für HB101(pN113), DSM 5763 für HB101(pN119) und DSM 5765 für HB101(pN124) hinterlegt. Der Transfervektor wird dann zusammen mit DNA des Wildtyp-Baculovirus in die Insektenzellen transfektiert. Die in den Zellen durch homologe Rekombination entstehenden rekombinanten Viren können dann nach bekannten Methoden identifiziert und isoliert werden. Eine Uebersicht über das Baculovirus-Expressionssystem und der dabei verwendeten Methoden findet man bei Luckow und Summers [82].

[0022] Exprimierte erfindungsgemäß Proteine können dann nach im Stand der Technik bekannten Methoden der Proteinchemie aus der Zellmasse oder den Kulturüberständen gereinigt werden.

[0023] Auf Grund der hohen Bindungsaffinität erfindungsgemässer TNF-BP für TNF (K_d -Werte in den Größenordnungen von 10^{-9} - 10^{-10} M) können diese oder Fragmente davon als Diagnostika zum Nachweis von TNF in Serum oder anderen Körperflüssigkeiten nach im Stand der Technik bekannten Methoden, beispielsweise in Postphasenbindungstests oder in Verbindung mit Anti-TNF-BP-Antikörpern in sogenannten "Sandwich"-Tests, eingesetzt werden.

[0024] Im übrigen können erfindungsgemässe TNF-BP einerseits zur Reinigung von TNF und andererseits zum Auffinden von TNF-Agonisten sowie TNF-Antagonisten nach im Stand der Technik bekannten Verfahren verwendet werden.

[0025] Die erfindungsgemässen Proteine sowie deren physiologisch verträgliche Salze, die nach im Stand der Technik bekannten Methoden hergestellt werden können, können auch zur Herstellung von pharmazeutischen Präparaten, vor allem solchen zur Behandlung von Krankheiten, bei deren Verlauf TNF involviert ist, verwendet werden. Dazu kann eine oder mehrere der genannten Verbindungen, falls wünschenswert bzw. erforderlich in Verbindung mit anderen pharmazeutisch aktiven Substanzen, mit den üblicherweise verwendeten festen oder flüssigen Trägermaterialien in bekannter Weise verarbeitet werden. Die Dosierung solcher Präparate kann unter Berücksichtigung der üblichen Kriterien in Analogie zu bereits verwendeten Präparaten ähnlicher Aktivität und Struktur erfolgen.

Referenz-Beispiel 1

Nachweis von TNF-bindenden Proteinen

[0026] Die TNF-BP wurden in einem Filtertest mit humanem radio-jodiertem 125 I-TNF nachgewiesen. TNF (46,47) wurde mit Na^{125}I (IMS40, Amersham, Amersham, England) und Iodo-Gen (#28600, Pierce Eurochemie, Oud-Beijerland, Niederlande) nach Fraker und Speck [48] radioaktiv markiert. Zum Nachweis der TNF-BP wurden isolierte Membranen der Zellen oder ihre solubilisierten, angereicherten und gereinigten Fraktionen auf angefeuchtete Nitrocellulose-Filter (0.45 μ , BioRad, Richmond, California, USA) aufgetragen. Die Filter wurden dann in Pufferlösung mit 1% entfettetem Milchpulver blockiert und anschliessend mit $5 \cdot 10^5$ cpm/ml 125 I-TNF α ($0.3\text{-}1.0 \cdot 10^8$ cpm/ μ g) in zwei Ansätzen mit und ohne Beigabe von 5 μ g/ml nicht-markiertem TNF α inkubiert, gewaschen und luftgetrocknet. Die gebundene Radioaktivität wurde autoradiographisch semiquantitativ nachgewiesen oder in einem γ -Counter gezählt. Die spezifische 125 I-TNF- α -Bindung wurde nach Korrektur für unspezifische Bindung in Anwesenheit von unmarkiertem TNF- α im Ueberschuss ermittelt. Die spezifische TNF-Bindung im Filtertest wurde bei verschiedenen TNF-Konzentrationen gemessen und nach Scatchard analysiert [33], wobei ein K_d -Wert von $\sim 10^{-9}$ - 10^{-10} M ermittelt wurde.

Referenz-Beispiel 2Zellextrakte von HL-60-Zellen

- 5 [0027] HL60 Zellen [ATCC-Nr. CCL 240] wurden in experimentellem Labormasstab in einem RPMI 1640-Medium [GIBCO-Katalog Nr. 074-01800], das noch 2 g/l NaHCO_3 und 5% fötales Kälberserum enthielt, in einer 5% CO_2 -Atmosphäre kultiviert und anschliessend zentrifugiert.
- 10 [0028] Zum Erzielen hoher Zelldichten in technischem Masstab wurde folgendermassen verfahren. Die Züchtung wurde in einem 75 l Airlifftermenter (Fa. Chemap, Schweiz) mit 58 l Arbeitsvolumen durchgeführt. Hierfür wurde das
- 15 Kassettenmembransystem "PROSTAK" (Millipore, Schweiz) mit einer Membranfläche von $0,32 \text{ m}^2$ (1 Kassette) in den äusseren Zirkulationskreislauf integriert. Das Kulturmedium (siehe Tabelle 1) wurde mit einer Watson-Marlow Pumpen TYP 603U, mit 5 l/min. umgepumpt. Nach einer Dampfsterilisation der Anlagen wobei das "PROSTAK" System im Autoklaven separat sterilisiert wurde, wurde die Fermentation mit wachsenden HL-60 Zellen aus einem 20 l Airlifftermenter (Chemap) gestartet. Die Zellzüchtung im Impff fermenter erfolgte im konventionellen Batchverfahren in dem Medium
- 20 gemäss Tabelle 1 und einem Startzelltiter von 2×10^5 Zellen/ml. Nach 4 Tagen wurde der HL60 Ansatz mit einem Titer von $4,9 \times 10^6$ Zellen/ml in den 75 l Fermenter überführt. Der pH-Wert wurde bei 7,1 und der pO_2 Wert bei 25% Sättigung gehalten, wobei der Sauerstoffeintrag durch eine mikroporöse Fritte erfolgte. Nach anfänglicher Batchfermentation wurde am 2. Tag die Perfusion bei einem Zelltiter von 4×10^6 Zellen/ml mit 30 l Mediumsaustausch Pro Tag gestartet. Auf der Filtratseite der Membran wurde das konditionierte Medium abgezogen und durch den Zulauf von frischem
- 25 Medium ersetzt. Das Zulaufmedium wurde wie folgt verstärkt: Primatone von 0,25% auf 0,35%, Glutamin von 5 mM auf 6 mM und Glucose von 4 g/l auf 6 g/l. Die Perfusionsrate wurde dann am 3. und 4. Tag auf 72 l Medium/Tag und am 5. Tag auf 100 l Medium/Tag erhöht. Nach 120 Stunden der kontinuierlichen Züchtung wurde die Fermentation beendet. Unter den gegebenen Fermentationsbedingungen erfolgte exponentielles Zellwachstum bis 40×10^6 Zellen/ml. Die Verdopplungszeit der Zellpopulation betrug bis 10×10^6 Zellen/ml 20-22 Stunden und stieg dann mit zunehmender Zelldichte auf 30-36 Stunden an. Der Anteil der lebenden Zellen lag während der gesamten Fermentationszeit bei 90-95%. Der HL-60 Ansatz wurde dann im Fermenter auf ca. 12°C heruntergekühlt und die Zellen durch Zentrifugation (Beckman-Zentrifuge [Modell J-6B, Rotor JS], 3000 rpm, 10 min., 4°C) geerntet.

Tabelle 1HL-60 MediumKomponentenKonzentrationenmg/l CaCl_2 (wasserfrei)

112,644

 $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$

20

 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ $0,498 \cdot 10^{-3}$ $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$

0,02

 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$

0,1668

KCl

336,72

 KNO_3

0,0309

 MgCl_2 (wasserfrei)

11,444

	MgSO ₄ (wasserfrei)	68,37
	NaCl	5801,8
5	Na ₂ HPO ₄ (wasserfrei)	188,408
	NaH ₂ PO ₄ •H ₂ O	75
	Na ₂ SeO ₃ •5H ₂ O	9,6•10 ⁻³
10	ZnSO ₄ •7H ₂ O	0,1726
	D-Glucose	4000
	Glutathion (red.)	0,2
15	Hepes-Puffer	2383,2
	Hypoxanthin	0,954
	Linolsäure	0,0168
	Liponsäure	0,042
20	Phenolrot	10,24
	Putrescin 2HCl	0,0322
	Na-Pyruvat	88
25	Thymidin	0,146
	Biotin	0,04666
	D-Ca-Pantothenat	2,546
	Cholinchlorid	5,792
30	Folsäure	2,86
	i-Inositol	11,32
	Niacinamid	2,6
35	Nicotinamid	0,0074
	para-Aminobenzoessäure	0,2
	Pyridoxal HCl	2,4124
	Pyridoxin HCl	0,2
40	Riboflavin	0,2876
	Thiamin HCl	2,668
	Vitamin B ₁₂	0,2782
45	L-Alanin	11,78
	L-Asparaginsäure	10
	L-Asparagin H ₂ O	14,362
50	L-Arginin	40
	L-Arginin HCl	92,6
	L-Aspartat	33,32

	L-Cystin 2HCl	62,04
	L-Cystein HCl•H ₂ O	7,024
5	L-Glutaminsäure	36,94
	L-Glutamin	730
	L-Glycin	21,5
10	L-Histidin	3
	L-Histidin HCl•H ₂ O	27,392
	L-Hydroxyprolin	4
15	L-Isoleucin	73,788
	L-Leucin	75,62
	L-Lysin HCl	102,9
20	L-Methionin	21,896
	L-Phenylalanin	43,592
	L-Prolin	26,9
	L-Serin	31,3
25	L-Threonin	53
	L-Tryptophan	11,008
	L-Tyrosin•2Na	69,76
30	L-Valin	62,74
	Penicillin/Streptomycin	100 U/ml
35	Insulin (human)	5 µg/ml
	Transferrin (human)	15 µg/ml
	Rinderserumalbumin	67 µg/ml
40	Primatone RL (Sheffield Products, Norwich NY, USA)	0,25%
	Pluronic F68	
	(Serva, Heidelberg, BRD)	0,01%
45	Fötales Kälberserum	0,3-3%

50 [0029] Das Zentrifugat wurde mit isotonem Phosphatpuffer (PBS; 0,2 g/l KCl, 0,2 g/l KH₂PO₄, 8,0 g/l NaCl, 2,16 g/l Na₂HPO₄ • 7H₂O), der mit 5% Dimethylformamid, 10 mM Benzamidin, 100 E/ml Aprotinin, 10 µM Leupeptin, 1 µM Pepstatin, 1 mM o-Phenanthrolin, 5 mM Jodacetamid, 1 mM Phenylmethylsulfonylfluorid versetzt war (im folgenden als PBS-M bezeichnet), gewaschen. Die gewaschenen Zellen wurden bei einer Dichte von 2,5 • 10⁸ Zellen/ml in PBS-M mit Triton X-100 (Endkonzentration 1,0%) extrahiert. Der Zellextrakt wurde durch Zentrifugation geklärt (15'000 x g, 1 Stunde; 100'000 x g, 1 Stunde).

Referenz-Beispiel 3Herstellung von monoklonalen (TNF-BP)-Antikörpern

- 5 [0030] Ein gemäß Referenz-Beispiel 2 erhaltener Zentrifugationsüberstand aus Kultivierung von HL60-Zellen im experimentellen Labormassstab wurde im Verhältnis 1:10 mit PBS verdünnt. Der verdünnte Überstand wurde bei 4°C auf eine Säule aufgetragen (Flussrate: 0,2 ml/min.), die 2 ml Affigel 10 enthielt (Bio Rad Katalog Nr. 153-6099), an das 20 mg rekombinantes humanes TNF- α [Pennica, D. et al. (1984) Nature 312, 724; Shirai, T. et al. (1985) Nature 313, 803; Wang, A.M. et al. (1985) Science 228, 149] gemäß den Empfehlungen des Herstellers gekoppelt worden war. Die
- 10 Säule wurde bei 4°C und einer Durchflussrate von 1 ml/min zuerst mit 20 ml PBS, das 0,1% Triton X 114 enthielt und danach mit 20 ml PBS gewaschen. So angereichertes TNF-BP wurde bei 22°C und einer Flussrate von 2 ml/min mit 4 ml 100 mM Glycin, pH 2.8, 0,1% Decylmaltosid eluiert. Das Eluat wurde in einer Centricon 30 Einheit [Amicon] auf 10 μ l konzentriert.
- [0031] 10 μ l dieses Eluates wurden mit 20 μ l vollständigem Freundschens Adjuvans zu einer Emulsion gemischt. Je 15 10 μ l der Emulsion wurden gemäß dem von Holmdahl, R. et al. [(1985), J. Immunol. Methods 83 379] beschriebenen Verfahren an den Tagen 0, 7 und 12 in eine hintere Fusspfote einer narkotisierten Balb/c-Maus injiziert.
- [0032] Am Tag 14 wurde die immunisierte Maus getötet, der popliteale Lymphknoten herausgenommen, zerkleinert und in Iscove's Medium (IMEM, GIBCO Katalog Nr. 074-2200), das 2 g/l NaHCO₃ enthielt, durch wiederholtes Pipettieren suspendiert. Gemäß einem modifizierten Verfahren von De St.Groth und Scheidegger [J. Immunol. Methods (1980), 35, 1] wurden 5x10⁷ Zellen des Lymphknotens mit 5x10⁷ P815-Maus-Myelomazellen (J.W. Stocker et al., Research Disclosure, 217, Mai 1982, 155-157), die sich in logarithmischem Wachstum befanden, fusioniert. Die Zellen wurden gemischt, durch Zentrifugation gesammelt und durch leichtes Schütteln in 2 ml 50% (v/v) Polyethylenglycol in IMEM bei Raumtemperatur resuspendiert und durch langsame Zugabe von 10 ml IMEM während 10 Minuten vorsichtigen Schüttelns verdünnt. Die Zellen wurden durch Zentrifugation gesammelt und in 200 ml vollständigem
- 25 Medium (IMEM + 20% fötales Kälberserum, Glutamin (2,0 mM), 2-Mercaptoethanol (100 μ M), 100 μ M Hypoxanthine, 0,4 μ M Aminopterin und 16 μ M Thymidine (HAT)) resuspendiert. Die Suspension wurde auf 10 Gewebekulturschalen, die jeweils 96 Vertiefungen enthielten, verteilt und ohne Wechsel des Mediums bei 37°C in einer Atmosphäre von 5% CO₂ und einer relativen Luftfeuchtigkeit von 98% 11 Tage lang inkubiert.
- [0033] Die Antikörper zeichnen sich aus durch ihre inhibierende Wirkung auf die TNF-Bindung an HL60-Zellen oder durch ihre Bindung an Antigen im Filtrertest gemäß Referenz-Beispiel 1. Zum Nachweis der biologischen Aktivität von anti-(TNF-BP)-Antikörpern wurde folgendes massenverfahren: 5x10⁶ HL60 oder U937-Zellen wurden in vollständigem RPMI 1640 Medium zusammen mit affinitätsgereinigten monoklonalen anti-(TNF-BP)-Antikörpern oder Kontrollantikörpern (d.h. solchen, die nicht gegen TNF-BP gerichtet sind) in einem Konzentrationsbereich von 1 ng/ml bis 10 μ g/ml inkubiert. Nach einer Stunde Inkubation bei 37°C wurden die Zellen durch Zentrifugation gesammelt und mit 4,5
- 35 ml PBS bei 0°C gewaschen. Sie wurden in 1 ml vollständigem RPMI 1640 Medium (Referenz-Beispiel 2), das zusätzlich 0,1% Natriumazid und ¹²⁵I-TNF α (10⁶ cpm/ml) mit oder ohne Beigabe von unmarkiertem TNF α (s.o.) enthielt, resuspendiert. Die spezifische Radioaktivität des ¹²⁵I-TNF α betrug 700 Ci/mmol. Die Zellen wurden 2 Stunden bei 4°C inkubiert, gesammelt und 4 mal mit 4,5 ml PBS, das 1% BSA und 0,001% Triton X 100 (Fluka) enthielt, bei 0°C gewaschen. Die an die Zellen gebundene Radioaktivität wurde in einem γ -Scintillations-zähler gemessen. In einem vergleichbaren Experiment wurde die zellgebundene Radioaktivität von Zellen, die nicht mit anti-(TNF-BP)-Antikörpern behandelt worden waren, bestimmt (ungefähr 10 000 cpm/5x10⁶ Zellen).

Referenz-Beispiel 445 Affinitätschromatographie

- [0034] Für die weitere Reinigung wurden jeweils ein gemäß Referenz-Beispiel 3 erhaltener monoklonaler anti-(55 kD TNF-BP)-Antikörper (2,8 mg/ml Gel), TNF α (3,0 mg/ml Gel) und Rinderserumalbumin (BSA, 8,8 mg/ml Gel) gemäß den Vorschriften des Herstellers kovalent an CNBr-aktivierte Sepharose 4B (Pharmacia, Uppsala, Schweden) gekoppelt. Der gemäß Referenz-Beispiel 2 erhaltene Zellextrakt wurde über die so hergestellten und in der folgenden Reihenfolge hintereinandergeschalteten Säulen geleitet: BSA-Sepharose-Vorsäule, Immunaффinitätssäule [Anti-(55 kD-TNF-BP)-Antikörper], TNF α -Ligand-Affinitätssäule. Nach vollständigem Auftrag wurden die beiden letztgenannten Säulen abgetrennt und einzeln für sich mit je 100 ml der folgenden Pufferlösungen gewaschen: (1) PBS, 1,0% Triton X-100, 10 mM Benzamidin, 100 E/ml Aprotinin; (2) PBS, 0,1% Triton X-100, 0,5M NaCl, 10 mM ATP, 10 mM Benzamidin, 100 E/ml Aprotinin; und (3) PBS, 0,1% Triton X-100, 10 mM Benzamidin, 100 E/ml Aprotinin. Sowohl die Immun- als auch die TNF α -Ligand-Affinitätssäule wurden dann mit 100 mM Glycin pH 2.5, 100 mM NaCl, 0,2% Decylmaltoside, 10 mM Benzamidin, 100 E/ml Aprotinin jede für sich eluiert. Die im Filtrertest gemäß Beispiel 1 aktiven Fraktionen jeder Säule wurden danach jeweils vereint und mit 1M Tris pH 8,0 neutralisiert.

[0035] Die so vereinten TNF-BP-aktiven Fraktionen der ImmunAffinitätschromatographie einerseits und der TNF α -LigandAffinitätschromatographie andererseits wurden zur weiteren Reinigung nochmals auf je eine kleine TNF α -Ligand-Affinitätssäule aufgetragen. Danach wurden diese beiden Säulen mit je 40 ml von (1) PBS, 1,0% Triton X-100, 10 mM Benzamidin, 100 E/ml Aprotinin, (2) PBS, 0,1% Triton X-100, 0,5M NaCl, 10 mM ATP, 10mM Benzamidin, 100 E/ml Aprotinin, (3) PBS, 0,1% Triton X-100, (4) 50 mM Tris PH 7.5, 150 mM NaCl, 1,0% NP-40, 1,0% Desoxycholat, 0,1% SDS, (5) PBS, 0,2% Decylmaltosid gewaschen. Anschliessend wurden die Säulen mit 100 mM Glycin pH 2.5, 100 mM NaCl, 0,2% Decylmaltosid eluiert. Fraktionen von 0,5 ml von jeder Säule wurden für sich gesammelt und die gemäss Filtertest (Referenz-Beispiel 1) aktiven Fraktionen von jeder Säule jeweils für sich vereint und in einer Centri-con-Einheit (Amicon, Molekulargewichts-Ausschluss 10'000) aufkonzentriert.

Referenz-Beispiel 5

Auftrennung mittels HPLC

[0036] Die gemäss Referenz-Beispiel 4 erhaltenen aktiven Fraktionen wurden gemäss ihrer unterschiedlichen Herkunft (Immun- bzw. Ligand-Affinitätschromatographie) jeweils für sich auf C1/C8 Umkehrphasen-HPLC-Säulen (ProRPC, Pharmacia, 5x20 mm), die mit 0,1% Trifluoressigsäure, 0,1% Octylglucosid equilibriert worden waren, aufgetragen. Die Säulen wurden dann mit einem linearen Acetonitril-Gradienten (0-80%) im gleichen Puffer bei einem Fluss von 0.5 ml/min eluiert. Fraktionen von 1,0 ml wurden von jeder Säule gesammelt und die aktiven Fraktionen von jeder Säule für sich vereint (Nachweis gemäss Referenz-Beispiel 1).

Referenz-Beispiel 6

Auftrennung mittels SDS-PAGE

[0037] Die gemäss Referenz-Beispiel 5 erhaltenen und gemäss Filtertest (Referenz-Beispiel 1) aktiven Fraktionen wurden durch SDS-PAGE gemäss [34] weiter aufgetrennt. Dazu wurden die Proben in SDS-Probenpuffer während 3 Minuten auf 95°C erhitzt und anschliessend auf einem 12% Acrylamid-Trenngel mit einem 5%igen Sammelgel elektrophoretisch aufgetrennt. Als Referenz zur Bestimmung der scheinbaren Molekulargewichte auf dem SDS-PAGE Gel wurden die folgenden Eichproteine verwendet: Phosphorylase B (97,4 kD), BSA (66,2 kD), Ovalbumin (42,7 kD), Carboanhydrase (31,0 kD), Soja Trypsin-Inhibitor (21,5 kD) und Lysozym (14,4 kD).

[0038] Unter den genannten Bedingungen wurden für Proben, die gemäss Referenz-Beispiel 4 durch TNF α -Ligandenaffinitätschromatographie von Immunaffinitätschromatographieeluat erhalten und durch HPLC gemäss Referenz-Beispiel 5 weiter aufgetrennt worden waren, zwei Banden von 55 kD und 51 kD sowie drei schwächere Banden von 38 kD, 36 kD und 34 kD erhalten. Diese Banden wurden in einem Mini Trans Blot System (BioRad, Richmond, California, USA) elektrophoretisch während 1 Stunde bei 100 V in 25 mM Tris, 192 mM Glycin, 20% Methanol auf eine PVDF-Membran (Immobilon, Millipore, Bedford, Mass. USA) transferiert. Danach wurde die PVDF-Membran entweder mit 0,15% Serva-Blau (Serva, Heidelberg, BRD) in Methanol/Wasser/Eisessig (50/40/10 Volumenteile) auf Protein gefärbt oder mit entfettetem Milchpulver blockiert und anschliessend zum Nachweis von Banden mit TNF-BP-Aktivität mit 125 I-TNF α gemäss den in Beispiel 1 beschriebenen Filtertestbedingungen inkubiert. Dabei zeigte sich, dass alle in der Proteinfärbung zur Darstellung gelangten Banden spezifisch TNF α banden. Alle diese Banden banden im Western Blot nach Towbin et al. [38] auch den gemäss Referenz-Beispiel 3 hergestellten monoklonalen Anti-55kD-TNF-BP-Antikörper. Dabei wurde ein gemäss dem in Referenz-Beispiel 1 beschriebenen Verfahren mit Na 125 I radioaktiv markierter, affinitätsgereinigter (Mausimmunglobulin-Sepharose-4B-Affinitätssäule) Kaninchen-anti-Maus-Immunglobulin-Antikörper zum autoradiographischen Nachweis dieses Antikörpers eingesetzt.

Referenz-Beispiel 7

Aminosäuresequenzanalyse

[0039] Zur Aminosäuresequenzanalyse wurden die gemäss Referenz-Beispiel 5 erhaltenen und gemäss Filtertest (Referenz-Beispiel 1) aktiven Fraktionen mittels der in Referenz-Beispiel 6 beschriebenen, nun jedoch reduzierenden, SDS-PAGE Bedingungen (SDS-Probenpuffer mit 125 mM Dithiothreitol) aufgetrennt. Es wurden die gleichen Banden wie gemäss Referenz-Beispiel 6 gefunden, die allerdings auf Grund der reduzierenden Bedingungen der SDS-PAGE im Vergleich zu Referenz-Beispiel 6 alle um etwa 1-2 kD höhere Molekulargewichte zeigten. Diese Banden wurden dann gemäss Referenz-Beispiel 6 auf PVDF-Membranen übertragen und mit 0,15% 35 Serva-Blau in Methanol/Wasser/Eisessig (50/40/10 Volumenteile) während 1 Minute gefärbt, mit Methanol/Wasser/Eisessig (45/48/7 Volumenteile) entfärbt, mit Wasser gespült, luftgetrocknet und danach ausgeschnitten. Bei sämtlichen Schritten wurden zur Vermeidung

5 dung von N-terminaler Blockierung die von Hunkapiller [34] angegebenen Bedingungen eingehalten. Zunächst wurden die gereinigten TNF-BP unverändert zur Aminosäuresequenzierung eingesetzt. Um zusätzliche Sequenzinformation zu erhalten, wurden die TNF-BP nach Reduktion und S-Carboxymethylierung [Jones, B.N. (1986) in "Methods of Protein Microcharacterisation", J.E. Shively, ed., Humana Press, Clifton NJ, 124-125] mit Bromcyan (Tarr, G.E. in "Methods of Protein Microcharacterisation", 165-166, op.cit.), Trypsin und/oder Proteinase K gespalten und die Peptide mittels HPLC nach bekannten Methoden der Proteinchemie aufgetrennt. So vorbereitete Proben wurden dann in einem automatisierten Gasphasen-Mikrosequenzier-Gerät (Applied Biosystems Modell 470A, ABI, Foster City, Calif., USA) mit einem on-line nachgeschalteten automatisierten HPLC PTH-Aminosäureanalysator (Applied Biosystems Modell 120, ABI s.o.) sequenziert, wobei die folgenden Aminosäuresequenzen bestimmt wurden:

10 1., Für die 55 kD-Bande (gemäss nichtreduzierender SDS-PAGE):

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile,
und

15 Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys

wobei X für einen Aminosäurerest steht, der nicht bestimmt werden konnte.

2., Für die 51 kD und die 38 kD-Banden (gemäss nichtreduzierender SDS-PAGE):

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu

Referenz-Beispiel 8

Bestimmung von Basen-Sequenzen von komplementärer DNA (cDNA)

25 [0040] Ausgehend von der Aminosäuresequenz gemäss Formel 1A wurden unter Berücksichtigung des genetischen Codes zu den Aminosäureresten 2-7 und 17-23 entsprechende, vollständig degenerierte Oligonucleotide in geeigneter Komplementarität synthetisiert ("sense" and "antisense" Oligonucleotide). Totale zelluläre RNA wurde aus HL60-Zellen isoliert [42, 43], und der erste cDNA-Strang durch Oligo-dT-Priming oder durch Priming mit dem "anti-sense" Oligonucleotid mittels eines cDNA-Synthese-Kits (RPN 1256, Amersham, Amersham, England) gemäss der
30 Anleitung des Herstellers synthetisiert. Dieser cDNA-Strang und die beiden synthetisierten degenerierten "sense" und "anti-sense" Oligonucleotide wurden in einer Polymerase-Kettenreaktion (PCR, Perkin Elmer Cetus, Norwalk, CT, USA gemäss Anleitung des Herstellers) dazu verwendet, die für die Aminosäure-Reste 8-16 (Formel 1A) codierende Basensequenz als cDNA-Fragment zu synthetisieren. Die Basensequenz dieses cDNA-Fragmentes lautet: 5'-AGGGAGAA-GAGAGATAGTGTGTGTC-3'. Dieses cDNA-Fragment wurde als Probe verwendet, um nach bekannten Verfahren
35 einen für das 55 kD TNF-BP codierenden cDNA-Klon in einer λ gt11-cDNA-Genbank von menschlicher Placenta zu identifizieren (42,43). Dieser Klon wurde dann nach üblichen Methoden aus dem λ -Vektor geschnitten und in die Plasmide pUC18 (Pharmacia, Uppsala, Sweden) und pUC19 (Pharmacia, Uppsala, Sweden) und in die M13mp18/M13mp19 Bacteriophagen (Pharmacia, Uppsala, Sweden) kloniert (42,43). Die Nukleotidsequenz dieses cDNA-Klons wurde mit einem Sequenase-Kit (U.S. Biochemical, Cleveland, Ohio, USA) nach den Angaben des Herstellers bestimmt. Die Nukleotidsequenz und die daraus abgeleitete Aminosäuresequenz für das 55 kD TNF-BP und dessen Signalpeptid (Aminosäure "-28" bis Aminosäure "0") ist in Figur 1 mittels der im Stand der Technik üblichen
40 Abkürzungen für Basen wie Aminosäuren dargestellt. Aus Sequenzvergleichen mit anderen, bereits bekannten Rezeptorproteinsequenzen lassen sich ungefähr 180 Aminosäuren enthaltende N-terminale wie 220 Aminosäure enthaltende C-terminale Domänen, die von einer nach den Sequenzvergleichen typischen Transmembran-Region von 19 Aminosäuren (in Figur 1 unterstrichen) getrennt werden, bestimmen. Hypothetische Glykosylierungsstellen sind in Figur 1
45 durch Sterne über der entsprechenden Aminosäure gekennzeichnet.

Referenz-Beispiel 9

Expression in COS 1-Zellen

50 [0041] Für die Expression in COS-Zellen wurden Vektoren ausgehend von dem Plasmid "pN11" konstruiert. Das Plasmid "pN11" enthält den effizienten Promotor und Enhancer des "major immediate-early" Gens des menschlichen Cytomegalovirus ("HCMV"; Boshart et al., Cell 41, 521-530, 1985). Hinter dem Promotor befindet sich eine kurze DNA-Sequenz, welche mehrere Restriktionsschnittstellen enthält, die nur einmal im Plasmid vorkommen ("Polylinker"), u.a. die Schnittstellen für HindIII, Ball, BamHI und PvuII (siehe Sequenz).

Pvu II

5' - AAGCTTGGCCAGGATCCAGCTGACTGACTGATCGCGAGATC - 3'
 3' - TTCGAACCGGTCCTAGGTGCGACTGACTGACTAGCGCTCTAG - 5'

Hinter diesen Schnittstellen befinden sich drei Translations-Stopcodons in allen drei Leserastern. Hinter der Polylinkersequenz befindet sich das 2. Intron und das Polyadenylierungssignal des Präproinsulins der Ratte (Lomedico et al., Cell 18, 545-558, 1979). Das Plasmid enthält ferner den Replikationsursprung des SV40 Virus sowie ein Fragment aus pBR322, das E. coli-Bakterien Ampicillin-Resistenz verleiht und die Replikation des Plasmids in E. coli ermöglicht.

[0042] Zur Konstruktion des Expressionsvektors "pN123" wurde dieses Plasmid "pN11" mit der Restriktionsendonuklease PvuII geschnitten und anschliessend mit alkalischer Phosphatase behandelt. Der dephosphorylierte Vektor wurde danach aus einem Agarosegel isoliert (V1). Die 5'-überhängenden Nukleotide des EcoRI-geschnittenen 1,3kb-Fragments der 55 kD TNF-BP-cDNA (siehe Referenz-Beispiel 8) wurden mit Hilfe von Klenow-Enzym aufgefüllt. Anschliessend wurde dieses Fragment aus einem Agarosegel isoliert (F1). Danach wurden V1 und F1 mittels T4-Ligase miteinander verbunden. E. coli HB101-Zellen wurden dann mit diesem Ligierungsansatz nach bekannten Methoden [42] transformiert. Mit Hilfe von Restriktionsanalysen und DNA-Sequenzierung nach bekannten Methoden [42] wurden Transformanten identifiziert, die mit einem Plasmid transformiert worden waren, welches das 1,3kb EcoRI-Fragment der 55 kD TNF-BP-cDNA in der für die Expression über den HCMV-Promotor korrekten Orientierung enthielt. Dieser Vektor erhielt die Bezeichnung "pN123".

[0043] Zur Konstruktion des Vektors "pK19" wurde folgendermassen verfahren. Ein DNA-Fragment, welches nur die für den extrazellulären Teil des 55 kD TNF-BP codierende cDNA enthält (Aminosäuren -28 bis 182 gemäss Figur 1) wurde mittels PCR-Technologie erhalten (Saiki et al., Science 230, 1350-1354, 1985, siehe auch Referenz-Beispiel 8). Die folgenden Oligonukleotide wurden, um die für den extrazellulären Teil des 55 kD TNF-BP codierende cDNA aus "pN123" zu amplifizieren, verwendet:

BAMHI

5' - CACAGGGATCCATAGCTGTCTGGCATGGGCCTCTCCAC - 3'

ASP718

3' - CGTGACTCCTGAGTCCGTGGTGTATTATCTCTAGACCATGGCCC - 5'

[0044] Durch diese Oligonukleotide wurden ebenfalls zwei Stopkodons der Translation hinter Aminosäure 182 eingeführt. Das so amplifizierte DNA-Fragment wurde mit BamHI und Asp718 geschnittene die hierbei entstandenen überstehenden Enden mit Hilfe des Klenow-Enzyms aufgefüllt und dieses Fragment anschliessend aus einem Agarosegel isoliert (F2). F2 wurde dann mit V1 ligiert und der gesamte Ansatz zur Transformation von E. coli HB101, wie bereits beschriebene verwendet. Transformanten, die mit einem Plasmid transformiert worden waren, welches das DNA-Fragment in der für die Expression über den HCMV-Promotor korrekten Orientierung enthielten, wurden mittels DNA-Sequenzierung (s.o.) identifiziert. Das daraus isolierte Plasmid erhielt die Bezeichnung "pK19".

[0045] Transfektion der COS-Zellen mit den Plasmiden "pN123" oder "pK19" wurde nach der von Felgner et al. veröffentlichten Lipofections-Methode (Proc. Natl. Acad. Sci. USA 84, 7413-7417, 1987) durchgeführt. 72 Stunden nach erfolgter Transfektion wurden die mit "pN123" transfizierten Zellen nach bekannten Methoden mit ¹²⁵I-TNFα auf Bindung analysiert. Das Resultat der Scatchard-Analyse [Scatchard, G., Ann. N.Y. Acad. Sci. 51, 660, 1949] der so erhaltenen Bindungsdaten (Figur 2A) ist in Figur 2B dargestellt. Die Kulturüberstände der mit "pK19" transfizierten Zellen wurden in einem "Sandwich"-Test untersucht. Dazu wurden PVC-Microtiterplatten (Dynatech, Arlington, VA, USA) mit 100 µl/Loch eines Kaninchen-anti-Maus Immunglobulins (10 µg/ml PBS) sensibilisiert. Anschliessend wurde die Platte gewaschen und mit einem anti-55 kD TNF-BP-Antikörper, der gemäss Referenz-Beispiel 3 durch seine Antigenbindung nachgewiesen und isoliert wurde, der aber die TNF-Bindung an Zellen nicht inhibiert, inkubiert (3 Stunden, 20°C). Die Platte wurde dann wieder gewaschen und über Nacht bei 4°C mit 100 µl/Loch der Kulturüberstände (1:4 verdünnt mit 1% entfetteter Milchpulver enthaltendem Puffer A: 50 mM Tris/HCl pH 7.4, 140 mM NaCl, 5 mM EDTA, 0,02% Na-Azid) inkubiert. Die Platte wurde entleert und mit ¹²⁵I-TNFα enthaltendem Puffer A (10⁶ cpm/ml, 100 µl/Loch) mit oder ohne Zusatz von 2 µg/ml unmarkiertem TNF während 2 Stunden bei 4°C inkubiert. Danach wurde die Platte 4 mal mit PBS gewaschen, die einzelnen Löcher wurden ausgeschnitten und in einem γ-Zähler gemessen. Die Resultate von 5 paral-

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lelen Transfektionen (Säulen # 2, 3, 4, 6 und 7), von zwei Kontroll-Transfektionen mit dem pN11-Vektor (Säulen # 1, 5) und von einer Kontrolle mit HL60-Zell-Lysat (Säule # 8) sind in Figur 3 dargestellt.

Referenz-Beispiel 10

Expression in Insektenzellen

[0046] Für die Expression in einem Baculovirus-Expressionssystem wurde von dem Plasmid "pVL941" (Luckow und Summers, 1989, "High Level Expression of Nonfused Foreign Genes with Autographa californica Nuclear Polyhedrosis virus Expression Vectors", Virology 170, 31-39) ausgegangen und dieses folgendermassen modifiziert. Es wurde die einzige EcoRI-Restriktionsschnittstelle in "pVL941" entfernt, indem das Plasmid mit EcoRI geschnitten und die überstehenden 5'-Enden mit Klenow-Enzym aufgefüllt wurden. Das hieraus erhaltene Plasmid pVL941/E- wurde mit BamHI und Asp718 verdaut und der Vektorrumpf anschliessend aus einem Agarosegel isoliert. Dieses Fragment wurde mit einem synthetischen Oligonukleotid der folgenden Sequenz ligiert:

```

          BamHI   EcoRI           Asp718
5' - GATCCAGAATTCATAATAG - 3'
3' -          GTCTTAAGTATTATCCATG - 5'

```

[0047] E. coli HB101 wurde mit dem Ligierungsansatz transformiert und Transformanten, die ein Plasmid enthielten, in welches das Oligonukleotid korrekt eingebaut worden war, wurden durch Restriktionsanalyse und DNA-Sequenzierung nach bekannten Methoden (s.o.) identifiziert; dieses Plasmid wurde "PNR704" genannt. Zur Konstruktion des Transfervektors "pN113" wurde dieses Plasmid "pNR704" mit EcoRI geschnitten, mit alkalischer Phosphatase behandelt und der so erzeugte Vektorrumpf (V2) anschliessend aus einem Agarosegel isoliert. Das wie oben mit EcoRI geschnittene 1,3 kb-Fragment der 55 kD TNF-BP-cDNA wurde mit Fragment V2 ligiert. Mit diesem Ligierungsansatz erhaltene Transformanten, die ein Plasmid enthielten, welches das cDNA-Insert in der korrekten Orientierung für die Expression über den Polyhedrinpromotor enthielten, wurden identifiziert (s.o.). Der daraus isolierte Vektor erhielt die Bezeichnung "PN113".

[0048] Zur Konstruktion des Transfervektors "pN119" wurde folgendermassen vorgegangen. Das 1,3 kb EcoRI/EcoRI-Fragment der 55 kD TNF-BP cDNA in dem "pUC19"-Plasmid (siehe Beispiel 8) wurde mit BamI verdaut und mit dem folgenden synthetischen Oligonukleotid ligiert:

```

          BamI           Asp718
5' - GCACCACATAATAGAGATCTGGTACCGGGAA - 3'
3' -          GTGTATTATCTCTAGACCATGGCCC - 5'

```

[0049] Mit dem obigen Adaptor werden zwei Stopcodons der Translation hinter Aminosäure 102 und eine Schnittstelle für die Restriktionsendonuklease Asp718 eingebaut. Nach erfolgter Ligation wurde der Ansatz mit EcoRI und Asp718 verdaut und das partielle 55 kD TNF-BP-Fragment (F3) isoliert. Weiterhin wurde das ebenfalls mit Asp718 und EcoRI geschnittene Plasmid "pNR704" mit F3 ligiert und der Ligierungsansatz in E. coli HB101 transformiert. Die Identifikation der Transformanten, welche ein Plasmid enthielten, in das die partielle 55 kD TNF-BP cDNA korrekt für die Expression integriert worden war, erfolgte wie bereits beschrieben. Das aus diesen Transformanten isolierte Plasmid erhielt den Namen "pN119".

[0050] Zur Konstruktion des Transfervektors "pN124" wurde folgendermassen vorgegangen. Das in Referenz-Beispiel 9 beschriebene, für den extrazellulären Teil des 55 kD TNF-BP codierende cDNA-Fragment wurde mit den angegebenen Oligonukleotiden mit Hilfe der PCR-Technologie, wie in Beispiel 9 beschriebene amplifiziert. Dieses Fragment wurde mit BamHI und Asp718 geschnitten und aus einem Agarosegel isoliert (F4). Das Plasmid "pNR704" wurde ebenfalls mit BamHI und Asp718 geschnitten und der Vektorrumpf (V4) wurde isoliert (s.o.). Die Fragmente V4 und F4 wurden ligiert, E. coli HB101 damit transformiert und der rekombinante Transfervektor "pN124" wurde, wie beschrieben, identifiziert und isoliert.

[0051] Zur Transfektion der Insektenzellen wurde folgendermassen vorgegangen. 3 µg des Transfervektors "pN113" wurden mit 1 µg DNA des Autographa californica-Nukleär-polyhedrosisvirus (AcMNPV) (EP 127839) in Sf9-Zellen (ATCC CRL 1711) transfiziert. Polyhedrin negative Viren wurden identifiziert und aus "Plaques" gereinigt [52]. Mit diesen rekombinanten Viren wurden wiederum Sf9 Zellen wie in [52] beschriebene infiziert. Nach 3 Tagen in Kultur wurden die infizierten Zellen auf Bindung von TNF mittels ^{125}I -TNF α untersucht. Dazu wurden die transfizierten Zellen mit einer Pasteurpipette von der Zellkulturschale abgewaschen und bei einer Zelldichte von 5×10^6 Zellen/ml Kulturmedium [52], das 10 ng/ml ^{125}I -TNF- α enthielt, sowohl in Anwesenheit wie Abwesenheit von 5 µg/ml nichtmarkiertem TNF- α resuspendiert und 2 Stunden auf Eis inkubiert. Danach wurden die Zellen mit reinem Kulturmedium gewaschen und die zellgebundene Radioaktivität in einem γ -Zähler gezählt (siehe Tabelle 2).

Tabelle 2

Zellen	Zellgebundene Radioaktivität pro 10^6 Zellen
nichtinfizierte Zellen (Kontrolle)	60 cpm
infizierte Zellen	1600 ± 330 cpm ¹⁾

¹⁾ Mittelwert und Standardabweichung aus 4 Experimenten

Beispiel 1

[0052] Analog zu dem in Referenz-Beispiel 9 beschriebenen Verfahren wurde das für den extrazellulären Bereich des 55 kDa TNF-BP codierende cDNA-Fragment, nun jedoch mit den folgenden Oligonukleotiden als Primer, in einer Polymerasen-Kettenreaktion amplifiziert: Oligonukleotid 1: Oligonukleotid 2:

Sst I

5'-ATA GAG CTC TGT GGT GCC TGA GTC CTC AG-3'

[0053] Dieses

Sst I

5'-TAC GAG CTC GGC CAT AGC TGT CTG GCA TG-3'

cDNA-Fragment wurde in den pCD4-Hy3-Vektor [DSM 5523; Europäische Patentanmeldung Nr. 90107393.2; Japanische Patentanmeldung Nr. 108967/90; US Patent Application Ser.No. 510773/90] ligiert, aus dem die CD4-cDNA über die Sst I-Restriktions-Schnittstellen herausgenommen worden war. SstI-Schnittstellen befinden sich in dem Vektor pCD4-Hy3 sowohl vor wie in dem CD4-Teilsequenzstück wie dahinter. Das Konstrukt wurde mittels Protoplastenfusion nach Oi et al. (Proc. Natl. Acad. Sci. USA **80**, 825-829, 1983) in J558-Myelomzellen (ATCC Nr. TIB6) transfiziert. Transfektanten wurden durch Zugabe von 5 µg/ml Mycophenolsäure und 250 µg/ml Xanthin (Traunecker et al., Eur. J. Immunol. **16**, 851-854 (1986)) in das Grundmedium (Dulbecco's modifiziertes Eagle's Medium, 10% fötales Kälberserum, $5 \times 10^{-5}\text{M}$ 2-Mercaptoethanol) selektioniert. Das von den transfizierten Zellen sekretierte Expressionsprodukt konnte mittels üblicher Methoden der Proteinchemie, z.B. TNF-BP-Antikörper-Affinitätschromatographie, gereinigt werden. Falls nicht bereits spezifisch angegeben, wurden zur Kultivierung der verwendeten Zelllinien, zum Klonieren, Selektionieren bzw. zur Expansion der klonierten Zellen Standardverfahren, wie z.B. von Freshney, R.I. in "Culture of Animal Cells", Alan R. Liss, Inc., New York (1983) beschrieben, verwendet.

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[0054]

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Patentansprüche

1. DNA-Sequenzen die eine Kombination aus zwei Teil-DNA-Sequenzen umfassen, wobei die eine Teilsequenz für lösliche TNF-bindende Fragmente von TNF-Rezeptoren kodiert und aus den folgenden DNA-Sequenzen auswählbar ist:

- (a) Fragmente von DNA-Sequenzen wie sie in Figur 1 dargestellt sind wie deren komplementären Stränge;
- (b) DNA-Sequenzen, die mit wie unter (a) definierten Fragmenten hybridisieren;
- (c) DNA-Sequenzen, die auf Grund der Entartung des genetischen Codes nicht mit Sequenzen, wie unter (a) und (b) definiert, hybridisieren, aber die für Polypeptide mit genau gleicher Aminosäuresequenz kodieren; und die andere Teil-Sequenz, für alle Domänen der konstanten Region der schweren Kette von humanen Immunglobulinen ausser der ersten Domäne der konstanten Region der schweren Kette von humanen Immunglobulinen der Klasse IgG kodiert.

2. DNA-Sequenzen gemäss Anspruch 1 wobei besagte humane Immunglobuline solche vom Typ Ig1 bzw. Ig3 sind.
3. Von DNA-Sequenzen gemäss einem der Ansprüche 1 oder 2 kodierte rekombinante Proteine.
4. Vektoren, die DNA-Sequenzen gemäss einem der Ansprüche 1 oder 2 enthalten und zur Expression der von diesen DNA-Sequenzen kodierten Proteine in prokaryotischen- wie eukaryotischen Wirtssystemen geeignet sind.
5. Prokaryotische- wie eukaryotische Wirtssysteme, die mit einem Vektor gemäss Anspruch 4 transformiert worden sind.
6. Wirtssysteme gemäss Anspruch 5, wobei diese Säuger- oder Insektenzellen sind.
7. Ein Verfahren zur Herstellung einer Verbindung gemäss Anspruch 3, das dadurch gekennzeichnet ist, dass man ein wie in Anspruch 5 oder 6 beanspruchtes transformiertes Wirtssystem in einem geeigneten Medium kultiviert und aus dem Wirtssystem selbst oder dem Medium diese Verbindung isoliert.
8. Proteine, herstellbar nach einem wie in Anspruch 7 beanspruchten Verfahren.
9. Pharmazeutische Präparate, insbesondere zur Behandlung von Krankheiten bei denen TNF involviert ist, wobei solche Präparate dadurch gekennzeichnet sind, dass sie eine oder mehrere Verbindungen gemäss Anspruch 3 oder 8 oder deren physiologisch verträgliche Salze, gewünschtenfalls in Kombination mit weiteren pharmazeutisch wirksamen Substanzen und/oder nicht-toxischen, inerten, therapeutisch verträglichen Trägermaterialien enthalten.
10. Verwendung einer Verbindung gemäss Anspruch 3 oder 8 zur Herstellung einer pharmazeutischen Zusammensetzung zur Behandlung von durch die toxische Wirkung von Endotoxinen hervorgerufenen Zuständen.
11. Verwendung einer Verbindung gemäss Anspruch 3 oder 8 zur Herstellung einer pharmazeutischen Zusammensetzung zur Behandlung pathologischer Zustände in denen TNF als Mediator von Immunantwort oder Entzündung beteiligt ist.
12. Verwendung einer Verbindung gemäss Anspruch 3 oder 8 als Diagnostikum zum Nachweis von TNF in Serum oder anderen Körperflüssigkeiten.
13. Verwendung einer Verbindung gemäss Anspruch 3 oder 8 zum Auffinden von TNF-Agonisten wie TNF-Antagoni-

sten.

Claims

- 5 1. DNA sequences which comprise a combination of two partial DNA sequences, with one of the partial sequences coding for soluble TNF-binding fragments of TNF receptors and being selected from the following DNA sequences:
 - (a) fragments of DNA sequences which are represented in Figure 1 as well as their complementary strands;
 - (b) DNA sequences which hybridize with the fragments defined under (a);
 - 10 (c) DNA sequences which, because of the degeneration of the genetic code, do not hybridize with sequences as defined under (a) and (b), but which code for polypeptides having exactly the same amino acid sequence; and the other partial sequence coding for all domains of the constant region of the heavy chain of human immunoglobulins except the first domain of the constant region of the heavy chain of human immunoglobulins of class IgG.
- 15 2. DNA sequences in accordance with claim 1, wherein said human immunoglobulins are those of the Ig1 or Ig3 type.
3. Recombinant proteins coded by DNA sequences in accordance with either claim 1 or claim 2,
- 20 4. Vectors which contain DNA sequences in accordance with either claim 1 or claim 2 and which are suitable for the expression in prokaryotic and eukaryotic host systems of proteins coded by these DNA sequences.
5. Prokaryotic and eukaryotic host systems which have been transformed with a vector in accordance with claim 4.
- 25 6. Host systems in accordance with claim 5, which are mammalian or insect cells.
7. A process for the production of a compound in accordance with claim 3, which is characterized by cultivating a host system transformed as claimed in claim 5 or 6 in a suitable medium and isolating said compound from the host system itself or from the medium.
- 30 8. Proteins, producible according to a process as claimed in claim 7.
9. Pharmaceutical preparations, especially for the treatment of illnesses in which TNF is involved, with such preparations being characterized in that they contain one or more compounds in accordance with claim 3 or 8 or their physiologically compatible salts; if desired in combination with additional pharmaceutically active substances and/or non-toxic, inert, therapeutically compatible carrier materials.
- 35 10. The use of a compound in accordance with claim 3 or 8 for the production of a pharmaceutical preparation for the treatment of conditions which are caused by the toxic effect of endotoxins.
- 40 11. The use of a compound in accordance with claim 3 or 8 for the production of a pharmaceutical preparation for the treatment of pathological conditions in which TNF is involved as a mediator of immune response or inflammation.
- 45 12. The use of a compound in accordance with claim 3 or 8 as a diagnostic for the identification of TNF in serum or other body fluids.
13. The use of a compound in accordance with claim 3 or 8 for the detection of TNF agonists and TNF antagonists.

Revendications

- 50 1. Séquences d'ADN qui comportent une combinaison de deux séquences partielles d'ADN, l'une des séquences partielles codant pour des fragments solubles, de récepteurs du TNF, se liant au TNF, et pouvant être choisie parmi les séquences d'ADN suivantes :
 - 55 (a) les fragments de séquences d'ADN telles que représentées sur la Figure 1, ainsi que leurs brins complémentaires,
 - (b) les séquences d'ADN qui s'hybrident aux fragments tels que définis en (a),
 - (c) les séquences d'ADN qui, du fait de la dégénérescence du code génétique, ne s'hybrident pas aux séquen-

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ces telles que définies en (a) et (b), mais codent pour des polypeptides ayant exactement la même séquence d'acides aminés ;

et l'autre séquence partielle code pour tous les domaines de la région constante de la chaîne lourde des immunoglobulines humaines, à l'exception des premiers domaines de la région constante de la chaîne lourde des immunoglobulines humaines de la classe des IgG.

2. Séquences d'ADN selon la revendication 1, dans lesquelles lesdites immunoglobulines humaines sont celles des types Ig1 ou Ig3.
3. Protéines recombinantes, codées par les séquences d'ADN selon l'une des revendications 1 ou 2.
4. Vecteurs qui contiennent les séquences d'ADN selon l'une des revendications 1 ou 2, et qui conviennent à l'expression des protéines codées par ces séquences d'ADN dans des systèmes hôtes tant procaryotes qu'eucaryotes.
5. Systèmes hôtes procaryotes ou eucaryotes, qui ont été transformés avec un vecteur selon la revendication 4.
6. Systèmes hôtes selon la revendication 5, qui sont des cellules de mammifères ou d'insectes.
7. Procédé de préparation d'un composé selon la revendication 3, caractérisé en ce qu'on cultive dans un milieu approprié un système hôte transformé tel que revendiqué dans les revendications 5 ou 6, et on isole ce composé du système hôte proprement dit, ou du milieu.
8. Protéines pouvant être préparées par un procédé tel que revendiqué dans la revendication 7.
9. Préparations pharmaceutiques, en particulier pour le traitement de maladies dans lesquelles est impliqué le TNF, ces préparations étant caractérisées en ce qu'elles contiennent un ou plusieurs composés selon la revendication 3 ou 8 ou leurs sels acceptables d'un point de vue physiologique, éventuellement en combinaison avec d'autres principes actifs pharmaceutiques et/ou des matériaux excipients non-toxiques, inertes, compatibles d'un point de vue thérapeutique.
10. Utilisation d'un composé selon la revendication 3 ou 8 pour préparer une composition pharmaceutique destinée au traitement d'états pathologiques provoqués par l'action toxique d'endotoxines.
11. Utilisation d'un composé selon la revendication 3 ou 8 pour préparer une composition pharmaceutique destinée au traitement d'états pathologiques dans lesquels le TNF participe en tant que médiateur de la réponse immunitaire ou de l'inflammation.
12. Utilisation d'un composé selon la revendication 3 ou 8 en tant que diagnostic pour détecter le TNF dans le sérum ou d'autres fluides corporels.
13. Utilisation d'un composé selon la revendication 3 ou 8 pour détecter tant des agonistes du TNF que des antagonistes du TNF.

Figur 1

-185 GAATTCGGGGGGGTTCAAGATCACTGGGACCAGGCCGTGATCTCTATGCCGGAGTCTCAA
 -125 CCCTCAACTGTCACCCCAAGGCACTTGGGACGTCCTGGACAGACCGAGTCCCGGGAAGCC
 -65 CCAGCACTGCCGCTGCCACACTGCCCTGAGCCCAAATGGGGGAGTGAGAGGCCATAGCTG
 -28.
 -30 MetGlyLeuSerThrValProAspLeuLeuLeuProLeuValLeuLeuGluLeu
 -5 TCTGGCATGGGCCTCTCCACCGTGCCCTGACCTGCTGCTGCCGCTGGTGCTGCTGGAGCTG
 +1
 -10 LeuValGlyIleTyrProSerGlyValIleGlyLeuValProHisLeuGlyAspArgGlu
 55 TTGGTGGGAATATACCCCTCAGGGGTTATTGGACTGGTCCCTCACCTAGGGGACAGGGAG

 10 LysArgAspSerValCysProGlnGlyLysTyrIleHisProGlnAsnAsnSerIleCys
 115 AAGAGAGATAGTGTGTGTCCCAAGGAAATATATCCACCCTCAAATAATTTCGATTTCG
 30 CysThrLysCysHisLysGlyThrTyrLeuTyrAsnAspCysProGlyProGlyGlnAsp
 175 TGTACCAAGTGCCACAAAGGAACCTACTTGTACAATGACTGTCCAGGCCCGGGGCAGGAT
 50 ThrAspCysArgGluCysGluSerGlySerPheThrAlaSerGluAsnHisLeuArgHis
 235 ACGGACTGCAGGGAGTGTGAGAGCGGCTCCTTCACCGCTTCAGAAAACCACTCAGACAC
 70 CysLeuSerCysSerLysCysArgLysGluMetGlyGlnValGluIleSerSerCysThr
 295 TGCCTCAGCTGCTCCAAATGCCGAAAGGAAATGGGTGAGGTGAGATCTCTTCTTGACA
 90 ValAspArgAspThrValCysGlyCysArgLysAsnGlnTyrArgHisTyrTrpSerGlu
 355 GTGGACCGGGACACCGTGTGTGGCTGCAGGAAGAACCAGTACCGGCATTATTGGAGTGAA

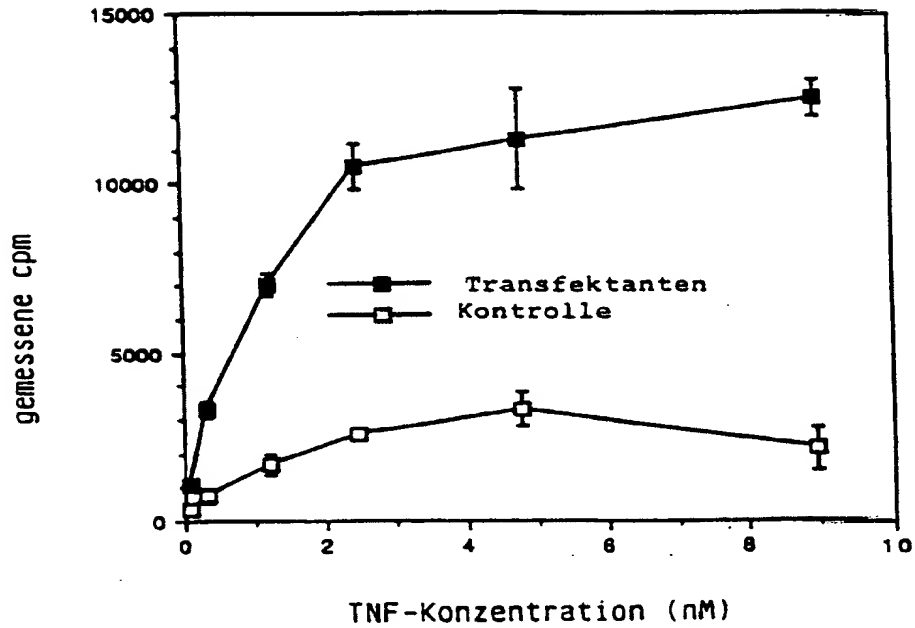
 110 AsnLeuPheGlnCysPheAsnCysSerLeuCysLeuAsnGlyThrValHisLeuSerCys
 415 AACCTTTTCCAGTGCTTCAATTGCAGCCTCTGCCTCAATGGGACCGTGACCTCTCCTGC
 130 GlnGluLysGlnAsnThrValCysThrCysHisAlaGlyPhePheLeuArgGluAsnGlu
 475 CAGGAGAAACAGAACACCGTGTGCACCTGCCATGCAGGTTTCTTTCTAAGAGAAAACGAG
 150 CysValSerCysSerAsnCysLysLysSerLeuGluCysThrLysLeuCysLeuProGln
 535 TGTGTCTCCTGTAGTAAGTGAAGAAAAGCCTGGAGTGCACGAAGTTGTGCCTACCCAG
 170 IleGluAsnValLysGlyThrGluAspSerGlyThrThrValLeuLeuProLeuValIle
 595 ATTGAGAATGTTAAGGGCACTGAGGACTCAGGCACCACAGTGCTGTTGCCGCTGGTCATT
 190 PhePheGlyLeuCysLeuLeuSerLeuLeuPheIleGlyLeuMetTyrArgTyrGlnArg
 655 TTCTTTGGTCTTTGCCTTTTATCCCTCCTCTTCATTGGTTTAATGTATCGCTACCAACGG
 210 TrpLysSerLysLeuTyrSerIleValCysGlyLysSerThrProGluLysGluGlyGlu
 715 TGGAAGTCCAAGCTCTACTCCATTGTTTGTGGGAAATCGACACCTGAAAAAGAGGGGGAG

 230 LeuGluGlyThrThrThrLysProLeuAlaProAsnProSerPheSerProThrProGly
 775 CTTGAAGGAACCTACTACTAAGCCCTGGCCCCAAACCAAGCTTCAGTCCCACTCCAGGC
 250 PheThrProThrLeuGlyPheSerProValProSerSerThrPheThrSerSerSerThr
 835 TTCACCCCCACCCTGGGCTTCAGTCCCGTGCCAGTTCACCTTCACCTCCAGCTCCACC
 270 TyrThrProGlyAspCysProAsnPheAlaAlaProArgArgGluValAlaProProTyr
 895 TATACCCCCGGTGACTGTCCCAACTTTCGGGCTCCCCGAGAGAGGTGCCACCACCCTAT
 290 GlnGlyAlaAspProIleLeuAlaThrAlaLeuAlaSerAspProIleProAsnProLeu
 955 CAGGGGGCTGACCCCATCCTTGCGACAGCCCTCGCCTCCGACCCCATCCCAACCCCTT

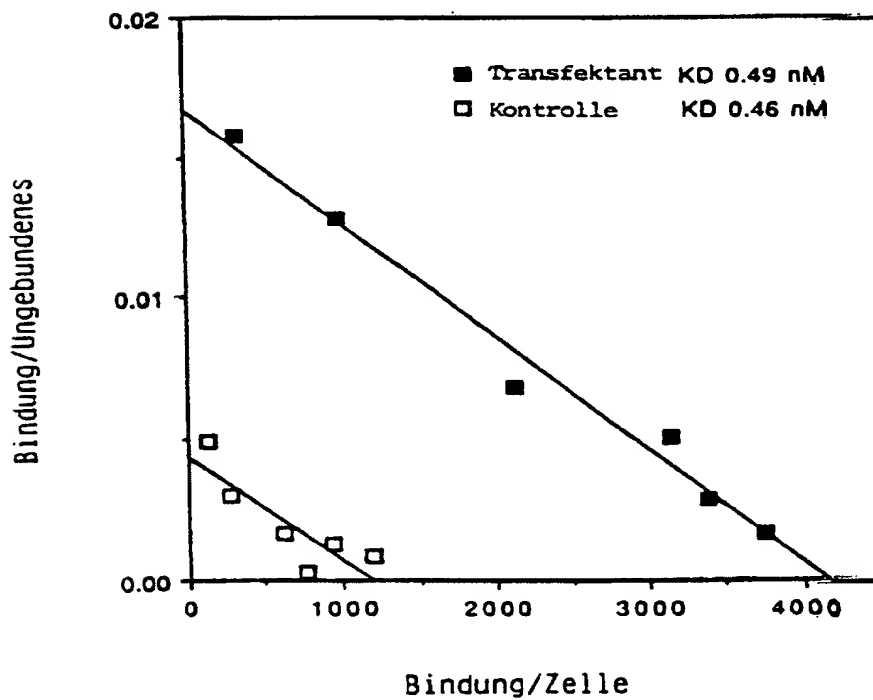
Figur 1a (Forts.)

310 GlnLysTrpGluAspSerAlaHisLysProGlnSerLeuAspThrAspAspProAlaThr
 1015 CAGAAGTGGGAGGACAGCGCCCAAGCCACAGAGCCTAGACACTGATGACCCCGCGACG
 330 LeuTyrAlaValValGluAsnValProProLeuArgTrpLysGluPheValArgArgLeu
 1075 CTGTACGCCGTGGTGGAGAACGTGCCCCGTTGCGCTGGAAGGAATTCGTGCGGCGCCTA
 350 GlyLeuSerAspHisGluIleAspArgLeuGluLeuGlnAsnGlyArgCysLeuArgGlu
 1135 GGGCTGAGCGACCACGAGATCGATCGGCTGGAGCTGCAGAACGGGCGCTGCCTGCGCGAG
 370 AlaGlnTyrSerMetLeuAlaThrTrpArgArgArgThrProArgArgGluAlaThrLeu
 1195 GCGCAATACAGCATGCTGGCGACCTGGAGCGGCGCACGCCGCGGCGAGGCCACGCTG
 390 GluLeuLeuGlyArgValLeuArgAspMetAspLeuLeuGlyCysLeuGluAspIleGlu
 1255 GAGCTGCTGGGACGCGTGCTCCGCGACATCGACCTGCTGGGCTGCCTGGAGGACATCGAG
 410 GluAlaLeuCysGlyProAlaAlaLeuProProAlaProSerLeuLeuArg
 1315 GAGGCGCTTTGCGGCCCCGCGCCCTCCCGCCCGCGCCAGTCTTCTCAGATGAGGCTGC
 1375 GCCCCTGCGGGCAGCTCTAAGGACCGTCCTGCGAGATCGCCTTCCAACCCCACTTTTTTC
 1435 TGAAAGGAGGGGTCTTGCAGGGGCAAGCAGGAGCTAGCAGCCGCCTACTTGGTGCTAAC
 1495 CCCTCGATGTACATAGCTTTTCTCAGCTGCCTGCGCGCCGCGACAGTCAGCGCTGTGCG
 1555 CGCGGAGAGAGGTGCGCCGTGGGCTCAAGAGCCTGAGTGGGTGGTTTTGCGAGGATGAGGG
 1615 ACGCTATGCCCTCATGCCCCGTTTTGGGTGTCTCACCAGCAAGGCTGCTCGGGGGCCCCCTG
 1675 GTTCGTCCCTGAGCCTTTTTACAGTGCATAAGCAGTTTTTTTTGTTTTTGTGTTTT
 1735 GTTTTGTGTTTTAAATCAATCATGTTACACTAATAGAACTTGGCACTCCTGTGCCCTCTG
 1795 CCTGGACAAGCACATAGCAAGCTGAAGTGTCTAAGGCAGGGGCGAGCACGGAACAATGG
 1855 GGCCTTCAGCTGGAGCTGTGGACTTTTGTACATACACTAAAATTCTGAAGTTAAAAAAA
 1915 AACCCGAATTC

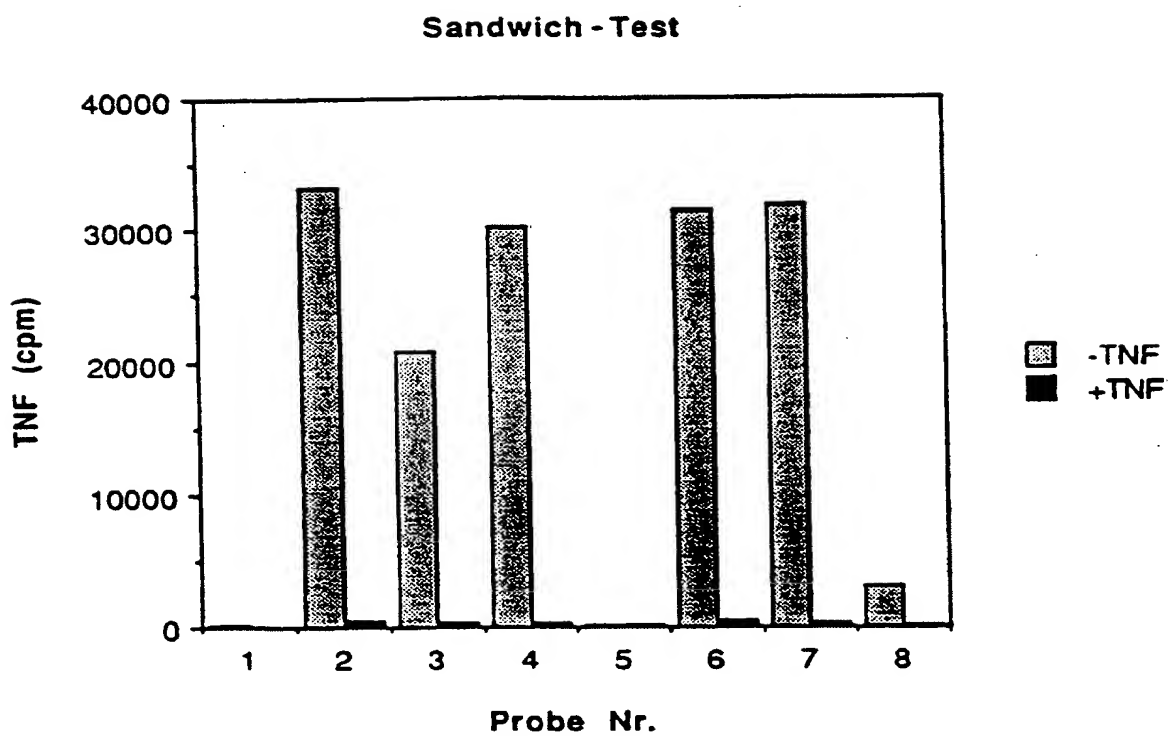
Figur 2A



Figur 2B



Figur 3



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Figure 4

1 SerAspSerValCysAspSerCysGluAspSerThrTyrThrGlnLeuTrpAsnTrpVal
 1 TCGGACTCCGTGTGTGACTCCTGTGAGGACAGCACATACACCCAGCTCTGGAACTGGGTT
 21 ProGluCysLeuSerCysGlySerArgCysSerSerAspGlnValGluThrGlnAlaCys
 61 CCCGAGTGCTTGAGCTGTGGCTCCCGCTGTAGCTCTGACCAGGTGGAACTCAAGCCTGC
 41 ThrArgGluGlnAsnArgIleCysThrCysArgProGlyTrpTyrCysAlaLeuSerLys
 121 ACTCGGGAACAGAACCCGCATCTGCACCTGCAGGCCCGGCTGGTACTGCGCGCTGAGCAAG
 61 GlnGluGlyCysArgLeuCysAlaProLeuProLysCysArgProGlyPheGlyValAla
 181 CAGGAGGGGGTGCCGGCTGTGCGCGCCGCTGCCGAAGTGCCGCCCGGGCTTCGGCGTGSCC
 81 ArgProGlyThrGluThrSerAspValValCysLysProCysAlaProGlyThrPheSer
 241 AGACCAGGAAGTGAACATCAGACGTGGTGTGCAGGCCCTGTGCCCGGGGACGTTCTCC
 101 AsnThrThrSerSerThrAspIleCysArgProHisGlnIleCysAsnValValAlaIle
 301 AACACGACTTCATCCACGGATATTTGCAGGCCCCACAGATCTGTACGTGGTGGCCATC
 121 ProGlyAsnAlaSerArgAspAlaValCysThrSerThrSerProThrArgSerMetAla
 361 CCTGGGAATGCAAGCAGGGATGCAGTCTGCACGTCCACGTCCCCACCCGGAGTATGGCC
 141 ProGlyAlaValHisLeuProGlnProValSerThrArgSerGlnHisThrGlnProSer
 421 CCAGGGGCAGTACACTTACCCAGCCAGTGTCCACACGATCCCAACACACGCAGCCAGT
 161 ProGluProSerThrAlaProSerThrSerPheLeuLeuProMetGlyProSerProPro
 481 CCAGAACCCAGCACTGCTCCAGCACCTCCTTCTGCTCCCAATGGGCCCCAGCCCCCA
 181 AlaGluGlySerThrGlyAspPheAlaLeuProValGlyLeuIleValGlyValThrAla
 541 GCTGAAGGGAGCACTGGCGACTTCGCTCTTCCAGTTGGACTGATTGTGGGTGTGACAGCC
 201 LeuGlyLeuLeuIleIleGlyValValAsnCysValIleMetThrGlnValLysLysLys
 601 TTGGGTCTACTAATAATAGGAGTGGTGAAGTGTGTCATCATGACCCAGGTGAAAAAGAG
 221 ProLeuCysLeuGlnArgGluAlaLysValProHisLeuProAlaAspLysAlaArgGly
 661 CCCTTGTGCCTGCAGAGAGAGCCAGGTGCCTCACTTGCTGCGGATAAGGCCCGGGGT
 241 ThrGlnGlyProGluGlnGlnHisLeuLeuIleThrAlaProSerSerSerSerSerSer
 721 ACACAGGGCCCCGAGCAGCAGCACCTGCTGATCACAGCGCCGAGCTCCAGCAGCAGCTCC
 261 LeuGluSerSerAlaSerAlaLeuAspArgArgAlaProThrArgAsnGlnProGlnAla
 781 CTGGAGAGCTCGGCCAGTGCCTTGGACAGAGGGGCGCCCACTCGGAACAGCCACAGGCA

Figur 4 (Fortsetzung)

281 ProGlyValGluAlaSerGlyAlaGlyGluAlaArgAlaSerThrGlySerSerAlaAsp
 841 CCAGGCGTGGAGGCCAGTGGGGCCGGGGAGGCCCGGGCCAGCACCAGGAGCTCAGCAGAT
 301 SerSerProGlyGlyHisGlyThrGlnValAsnValThrCysIleValAsnValCysSer
 901 TCTTCCCCTGGTGGCCATGGGACCCAGGTCAATGTACCTGCATCGTGAACGTCTGTAGC
 321 SerSerAspHisSerSerGlnCysSerSerGlnAlaSerSerThrMetGlyAspThrAsp
 961 AGCTCTGACCACAGCTCACAGTGCTCCTCCCAAGCCAGCTCCACATGGGAGACACAGAT
 341 SerSerProSerGluSerProLysAspGluGlnValProPheSerLysGluGluCysAla
 1021 TCCAGCCCCCTCGGAGTCCCCGAGGAGGAGGAGGTCCCCTTCTCCAGGAGGATGTGCC
 361 PheArgSerGlnLeuGluThrProGluThrLeuLeuGlySerThrGluGluLysProLeu
 1081 TTTGGTCACAGCTGGAGACGCCAGAGACCCTGCTGGGGAGCACCGAAGAGAGCCCCCTG
 381 ProLeuGlyValProAspAlaGlyMetLysProSer
 1141 CCCCTTGGAGTGCCTGATGCTGGGATGAAGCCAGTTAACCAGGCCGGTGTGGGCTGTGT
 1201 CGTAGCCAGGTGGCTGAGCCCTGGCAGGATGACCCTGCGAAGGGGCCCTGGTCCTTCCA
 1261 GGCCCCCACCCTAGGACTCTGAGGCTCTTTCTGGGCCAAGTTCCTCTAGTGCCCTCCAC
 1321 AGCCGCAGCCTCCCTCTGACCTGCAGGCCAAGAGCAGAGGCAGCGAGTTGTGGAAAGCCT
 1381 CTGCTGCCATGGCGTGTCCCTCTCGGAGGGCTGGCTGGGCATGGACGTTCCGGGGCATGCT
 1441 GGGGCAAGTCCCTGAGTCTCTGTGACCTGCCCCGCCAGCTGCACCTGCCAGCCTGGCTT
 1501 CTGGAGCCCTTGGGTTTTTTGTTTGTGTTTGTGTTTGTGTTTGTGTTTCTCCCCCTGGGC
 1561 TCTGCCAGCTCTGGCTTCCAGAAAACCCAGCATCCTTTTCTGCAGAGGGGCTTTCTGG
 1621 AGAGGAGGGATGCTGCCTGAGTCACCCATGAAGACAGGACAGTGCTTCAGCCTGAGGCTG
 1681 AGACTGCGGGATGGTCCTGGGGCTCTGTGCAGGGAGGAGGTGGCAGCCCTGTAGGGAAAG
 1741 GGGTCCTTCAAGTTAGCTCAGGAGGCTTGGAAAGCATCACCTCAGGCCAGGTGCAGTGGC
 1801 TCACGCCTATGATCCCAGCACTTTGGGAGGCTGAGGCGGGTGGATCACCTGAGGTTAGGA
 1861 GTTCGAGACCAGCCTGGCCACATGGTAAACCCCATCTCTACTAAAAATACAGAAATTA
 1921 GCCGGGCGTGGTGGCGGGCACCTATAGTCCCAAGCTACTCAGAAGCCTGAGGCTGGGAAT
 1981 CGTTTGAACCCGGGAGCGGAGGTTGCAGGGAGCCGAGATCACGCCACTGCACTCCAGCC
 2041 TGGGCGACAGAGCGAGAGTCTGTCTCAAAAGAAAAAAAAAAGCACCGCCTCCAAATGCT
 2101 AACTTGTCTTTTGTACCATGGTGTGAAGTCAGATGCCAGAGGGGCCAGGCAGGCCAC
 2161 CATATTCAGTGCTGTGGCCTGGGCAAGATAACGCACTTCTACTAGAAATCTGCCATTT
 2221 TTTAAAAAGTAAGTACCACTCAGGCCAACAGCCAACGACAAAGCCAACTCTGCCAGC
 2281 CACATCCAAACCCCCACCTGCCATTTGCACCTCCGCCTTCACTCCGGTGTGCTGCAG

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54 TNF-binding Proteins

57 The present invention relates to non-soluble proteins as well as to their soluble and non-soluble fragments, which bind TNF, in homogeneous form, as well as their physiologically compatible salts, especially such proteins with a molecular mass of 55 or 75 kDa (non-reducing SDS-PAGE conditions), processes for isolation of such protein antibodies against such proteins, DNA sequences, which are coded for non-soluble proteins as well as for their soluble or non-soluble fragments, which bind TNF, as well as those encoded for proteins, consisting in one part of a soluble fragment that binds TNF and in another part of all domains save the first of the constant region of the heavy chain of human immunoglobulins and the recombinant proteins thus encoded as well as processes for their production by means of transformed prokaryotic as well as eukaryotic cells.

TNF-BINDING PROTEINS

The Tumor Necrosis Factor α (TNF α , also cachectin), discovered because of its hemorrhagic-necrotizing effect on certain tumors, and lymphotoxin (TNF β) are two closely related peptide factors [3] of the class of lymphokines/cytokines, which will both from now on be designated as TNF [see survey papers 2 and 3]. TNF disposes over a broad cellular performance spectrum. For instance TNF has an inhibiting or cytotoxic effect on a series of tumor cell lines [2,3], stimulates the proliferation of fibroblasts and the phagocytizing/cytotoxic activity of myeloid cells [4,5,6], induces adhesion molecules in endothelial cells or has an inhibiting effect on endothelium [7,8,9,10], inhibits the synthesis of specific enzymes in adipocytes [11] and induces the expression of histocompatibility antigens [12]. Some of these TNF effects are achieved via induction from other factors or through synergistic effects with other factors, such as interferons or interleukins [13-16], for instance.

TNF is involved in a series of pathological conditions, for instance in shock conditions during meningococcal sepsis [17], during development of autoimmune glomerulonephritis in mice [18] or in case of cerebral malaria in mice [19] and humans [41]. Generally speaking, the toxic effects of endotoxin appear to be transmitted through TNF [20]. Furthermore, TNF can, just like interleukin-1, bring on fever [39]. Based on the pleiotropic functional characteristics of TNF it can be assumed that TNF is a participant in interaction with other cytokines in a whole series of further pathological conditions as mediator of immune response, inflammation or other processes.

~~These biological effects are transmitted through TNF via specific receptors,~~
with TNF α and TNF β both binding to the same receptors, according to the present

state of knowledge [21]. Different types of cells are differentiated by the number of TNF receptors [22,23,24]. Such quite generally considered TNF binding proteins (TNF-BP) were verified through covalent binding to radioactive marked TNF [24-29], with the following apparent molecular masses of the obtained TNF/TNF-BP complexes having been determined: 95/100 kDa and 75 kDa [24], 95 kDa and 75 kDa [25], 138 kDa, 90 kDa, 75 kDa, and 54 kDa [26]. 100 ± 5 kDa [27], 97 kDa and 70 kDa [28] and 145 kDa [29]. By means of anti-TNF-antibody-immunoaffinity chromatography and preparative SDS-polyacrilamide gel electrophoresis (SDS-PAGE) such a TNF/TNF-BP complex could be isolated [27]. The reductive cleavage of this complex and the subsequent SDS-PAGE analysis resulted in several bands, which were not tested for TNF binding activity, however. Since the specific conditions, which must be employed for cleavage of the complex, lead to an inactivation of the binding protein [31], the latter was made impossible. The enrichment of soluble TNF-BP from human serum or urine by means of ion exchange chromatography and gel filtration (molecular masses in the range of 50 kDa) was described by Olsson et al. [30].

Brockhaus et al. [32] obtained an enriched TNF-BP preparation through TNF α -ligandaffinity chromatography and HPLC from membrane extracts of HL60 cells, which was in turn used as antigen preparation for the production of monoclonal antibodies against TNF-BP. Through use of such an immobilized antibody (immunoaffinity chromatography) an enriched preparation of TNF-BP was obtained by means of TNF α ligand-affinity chromatography and HPLC by Loetscher and Brockhaus [31] from an extract of human placenta, which exhibited a strong wide band at 35 kDa, a weak band at about 40 kDa and a very weak band in the region between 55 kDa and 60 kDa, during the SDS-PAGE analysis. Moreover the gel exhibited a background smear in the region between 33 kDa and 40 kDa. The meaning of the protein bands thus obtained was not clear, however, in view of the heterogeneity of the initial material used (placenta tissue; material combined from several placentas).

The subject of the present invention is non-soluble proteins, i.e., for instance membrane proteins resp. so-called receptors and their soluble or non-soluble fragments, which bind TNF (TNF-BP), in homogeneous form, as well as their physiologically compatible salts. Preferred are those proteins which are characterized, according to SDS-PAGE under non-reducing conditions, by apparent molecular masses of about 55 kDa, 51 kDa, 38 kDa, 36 kDa, 36 kDa and 34 kDa resp. 75 kDa and 65 kDa particularly those with about 55 kDa and 75 kDa. Additionally preferred are those proteins, which are characterized by at least one of the following amino-acid part sequences:

(IA) Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile

(IB) Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys

(IIA) Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu

(IIB) Val-Phe-Cys-Thr

(IIC) Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala

(IID) Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys

(IIE) Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu

(IIF) Leu-Cys-Ala-Pro

(IIG) Val-Pro-His-Leu-Pro-Ala-Asp

(IIH) Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro where X stands for an amino acid residue that could not be determined unequivocally.

In the state of the technology TNF-BP have already been characterized by an N-terminal partial sequence [European Patent Application Nr. 308 378], with this sequence differing from the N-terminal partial sequence according to the invention according to formula (IA). The TNF binding proteins described in the state of the technology relate by the way to soluble i.e. not membrane-bound TNF-BP isolated from urine and not to membrane-bound, i.e. non-soluble, TNF-BP.

Also subject of the present application are processes for isolation of the TNF-BP according to the invention. These processes are characterized in that basically the following steps of purification are being carried out sequentially: production of a cell extract or tissue extract, immunoaffinity chromatography and/or simple or multiple ligandaffinity chromatography, high pressure liquid chromatography (HPLC) and preparative SDS-polyacrilamide gel electrophoresis (SDS-PAGE). The combination of the individual purification steps known from the state of current technology is essential for the success of the process according to the invention, with individual steps within the scope of the task to be solved having been modified and improved. For instance the combined immunoaffinity chromatography/TNF α -ligandaffinity chromatography step, initially used for enrichment of TNF-BP through human placenta [31], was changed in that a BSA-Sepharose 4B - precolumn was used. This precolumn was put in series with the immunoaffinity column and followed by the ligandaffinity column for application of the cell

extract or membrane extract. After application of the extract the two columns mentioned last were decoupled, each eluated separately and the TNF-BP active fractions were purified once more through a ligandaffinity column. Essential for the carrying out of the reverse phase HPLC step according to the invention is the use of a solvent mixture containing a detergent.

Furthermore a technical process to obtain high cell densities of mammalian cells, from which TNF-BP can be isolated, is also a subject of the present invention. Such a process is characterized in that a medium, which was developed for the specific growth requirements of the cell line used, was employed in combination with a perfusion instrument as described in detail in Example 2, for instance. By means of such a process cell densities for HL-60 cells may be increased to as much as 20 times higher than usual.

In addition the present invention relates to DNA sequences, which are coded for proteins that bind TNF and for their soluble and non-soluble fragments. By that we understand for instance DNA sequences that are coded for non-soluble proteins that bind TNF, or for their soluble and non-soluble fragments, with such DNA sequences being selectable from the following:

- (a) DNA sequences, as shown in Fig.1 or Fig.4, as well as their complete strands, or those that include these sequences;
- (b) DNA sequences that hybridize with sequences defined in (a) or with their fragments;
- (c) DNA sequences that do not hybridize with sequences as defined in (a) and (b) because of degeneration of the genetic code, but which code for polypeptides with exactly the same amino-acid sequence.

That means that the present invention includes not only allelic variants but also such DNA sequences that result from deletions, substitutions and additions of one or more nucleotides of the sequences shown in Fig.1 resp. Fig.4, with the thus coded proteins being at all times TNF-BP. A sequence resulting from such a

deletion is described in SCIENCE 248, 1019-1023, (1990), for instance.

Preferred are such DNA sequences that are coded for such a protein with an apparent molecular mass of about 55 kDa, with the sequence shown in Fig.1 being especially preferred as are sequences that are coded for non-soluble or soluble fragments of such proteins. A DNA sequence that is, for instance, coded for such a non-soluble protein fragment stretches from nucleotide -185 to 1122 of the sequence shown in Fig.1. DNA sequences that are coded for soluble protein fragments are, for instance, those that stretch from nucleotide -185 to 633 resp. from nucleotide -14 to 633 of the sequence shown in Fig.1. Also preferred are DNA sequences that are coded for a protein of about 75/65 kDa, with preference for those containing the partial cDNA sequences shown in Fig.4.

Especially preferred DNA sequences in this case are the sequences of the open reading frame from nucleotide 2 to 1177. The peptides IIA, IIC, IIE, IIF, IIG and IIH are encoded by the partial cDNA sequence in Fig.4, where the slight deviations in the experimentally determined amino acid sequences from the sequence derived from cDNA are most probably caused by the smaller resolution of gas phase sequencing. Also preferred are DNA sequences, which are coded for non-soluble as well as soluble fragments of TNF binding proteins with an apparent molecular mass of 75 kDa/65 kDa. DNA sequences for such soluble fragments can be determined based on the hydrophilia profiles of the amino sequences derived from nucleic acids coded for such non-soluble TNF-BP.

The invention relates furthermore to DNA sequences, which comprise a combination of two partial DNA sequences, with one partial sequence being coded for such soluble fragments of non-soluble proteins which bind TNF (above) while the other partial sequence is coded for all the domains, save the first domain of the constant region of the heavy chain of human immunoglobulins, like IgG, IgA, IgM resp. IgE.

The present invention relates naturally also to recombinant proteins encoded by such DNA sequences. It is self-evident that such proteins, in whose amino acid sequences amino acids have been exchanged, for instance by means of directed mutagenesis, in such a way that the activity of the TNF-BP or their fragments, specifically the binding of TNF or the interaction with other membrane components involved in the signal transmission, were changed or retained in a desired manner, are also included. Amino acid exchanges in proteins and peptides, which in general do not change the activity of such molecules, are known in the current state of technology and have been described, for instance, by H. Neurath and R.L. Hill in "The Proteins" (Academic Press, New York 1979, see in particular Fig.6, page 14). The exchanges occurring most frequently are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala,Val, Ser/Gly Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn,

Leu/Ile, Leu Val, Ala/Glu, Asp/Gly, as well as in reverse direction. The present invention relates further to vectors that contain DNA sequences according to the invention and are suitable for the transformation of suitable prokaryotic as well as eukaryotic host systems, with such vectors being preferred whose use leads to the expression of the proteins encoded by the DNA sequences according to the invention. Finally the invention relates also to prokaryotic as well as eukaryotic host systems transformed with such vectors, such as processes for production of recombinant combinations through cultivation of such host systems and subsequent isolation of these combinations from the host systems themselves or from their culture supernatants.

Also subject of the present invention are pharmaceutical preparations, which contain at least one of these TNF-BP or their fragments, if so desired in combination with additional pharmaceutically active substances and/or non-toxic, inert, therapeutically compatible carrier materials.

The present invention relates finally to the use of such TNF-BP on the one hand for production of pharmaceutical preparations, resp. on the other hand to the treatment of diseases, preferably those in the course of which TNF is involved.

The starting material for the TNF-BP according to the invention are in general cells that contain such TNF-BP in membrane-bound form and which are generally available to the specialist without any restrictions, like for instance HL60- [ATCC Nr. CCL 240], U 937- [ATCC Nr. CRL 1593], SW 480- [ATCC Nr. CCL 228] and HEp2 cells [ATCC Nr. CCL 23]. These cells may be cultivated according to known methods of the current state of technology [40] or for the attainment of high cell densities by means of the already in general and in detail described process for HL60 cells in example 2. TNF-BP may then be extracted according to known methods of the current state of technology by means of suitable detergents, for instance Triton X-114, 1-O-n-octyl- β -D-glucopyranoside (octylglucoside), or 3-[(3-cholylamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), particularly

by means of Triton X-100, from the cells that have been centrifuged off the medium and washed. The usually employed methods of identification for TNF-BP, for instance a polyethylene glycol-induced precipitation of the ^{125}I -TNF/TNF-BP complex [27], in particular filter binding tests with radioactively marked TNF according to Example 1, may be used. The current state of technology methods generally employed for purification of proteins, particularly of membrane proteins, such as ion exchange chromatography, gel filtration, affinity chromatography, HPLC and SDS-PAGE, may be used for the recovery of the TNF-BP according to the invention. Particularly preferred methods for the production of TNF-BP according to the invention are affinity chromatography, particularly with TNF- α as the ligand tied to the solid phase and immunoaffinity chromatography, HPL and SDS-PAGE. The elution of TNF-BP bands separated by means of SDS-PAGE can occur according to known methods of protein chemistry, for instance by means of electro-elution according to Hunkapiller et al. [34], with the electro dialysis times listed there to be doubled in general according to the present state of knowledge. Still remaining traces of SDS may then be removed according to Bosserhoff et al. [50].

Having been thus purified the TNF-BP may be characterized by means of the methods of peptide chemistry known at the current state of technology, as for instance N-terminal amino acid sequencing or enzymatic as well as chemical peptide cleavage. Fragments obtained through enzymatic or chemical cleavage may be separated by familiar methods, like HPLC for instance and then be subject of N-terminal sequencing themselves. Such fragments, which still bind TNF, may be identified by means of the methods of identification for TNF-BP listed above and are also a subject of the present invention.

Starting with the information about amino acid sequences or about the DNA sequences as well as amino acid sequences shown in Fig.1 and Fig.4 thus obtainable suitable oligonucleotides may be produced with methods known at the current state of technology while observing the genetic code degeneration [51]. With them and by again using well known methods of molecular biology [42,43] cDNA banks or genomic DNA banks may be scanned for clones containing nucleic acid sequences encoded for TNF-BP. In addition cDNA fragments may be cloned by means of the polymerase chain reaction (PCR)[49] by inserting completely degenerated and due to their complementarity suitable oligonucleotides that come from separate, relatively short sections of the amino acid sequence and by observing the genetic code, as "primers", which enables amplification and identification of the fragment that lies between these two sequences. Determination of the nucleotide sequence of such a fragment makes possible an independent determination of the amino acid sequence of the protein fragment for which it is encoded. The cDNA fragments obtainable through PCR may also be used, as already described for the oligonucleotides proper, by means of known methods, for the search for clones encoded for TNF-BP and containing nucleic acid sequences from cDNA banks resp. genomic DNA banks. Such nucleic acid sequences can then be sequenced according to known methods [42]. Based on the sequences thus determined as well as on those already known for certain receptors, such partial sequences which are encoded for soluble TNF-BP.

fragments, may be determined and cut out from the total sequence by means of known methods [42].

The total sequence or such partial sequences may then be integrated by means of known methods into vectors described in the current state of technology for their multiplication and as well as expression in prokaryotes [42]. Suitable prokaryotic host organisms are for instance gram-negative as well as gram-positive bacteria, like for instance *E. coli* strains, like *E. coli* HB 101 [ATCC Nr. 33 694] or *E. coli* W3110 [ATCC Nr. 27 325] or *B. subtilis* strains.

Furthermore nucleic acid sequences according to the invention, which are encoded for TNF-BP as well as for TNF-BP fragments, may be integrated into suitable vectors for proliferation as well as expression in eukaryotic host cells, like for instance yeast, insect cells and mammal cells, by means of known methods. Expression of such sequences occurs preferably in mammal as well as in insect cells.

A typical expression vector for mammal cells contains an efficient promoter element, for achievement of a good transcription rate, the DNA sequence to be expressed and signals for an efficient termination and polyadenylation of the transcript. Additional elements that can be used are "enhancers", which lead to further amplified transcription and sequences, which may for instance effect a longer biological half-life for the mRNA. For the expression of nucleic acid sequences in which the endogene sequence piece encoded for a signal peptide is missing, vectors may be used that contain such suitable sequences encoded for signal peptides of other known peptides. See for instance the vecotr pLJ268 described by Cullen, B.R. in Cell 46, 973-982(1986) or also by Sharms, S. et al. in "Current Communications in Molecular Biology", ed. by Gething, M.J. Cold Spring Harbor Lab.(1985), pages 73-78.

Most of the vectors used for a transient expression of a specific DNA sequence ~~in mammal cells contain the replication origin of the SV40 virus. In cells that~~ express the T-antigen of the virus, (for instance COS cells), these vectors pro-

liferate strongly. But a temporary expression is not limited to COS cells. In principle any mammal cell may be used for that purpose. Signals that can effect a strong transcription are, for instance, the early and late promoters of SV40, the promoter and enhancer of the "major immediate-early" gene of HCMV (human cytomegalovirus), the LTR's ("long terminal repeats") of retroviruses, like for instance RSV, HIV and MMTV. But signals from cellular genes, like for instance the promoters of the actin genes and collagenase genes, can be used.

Alternatively, stable cell lines that have integrated the specific DNA sequence in the genome (chromosome), may be obtained. For that the DNA sequence is co-transfected together with a selectable marker, for instance neomycin, hygromycin, dihydrofolate-reductase (dhfr) or hypoxanthine-guanine-phosphoribosyltransferase (hgbt).

The DNA sequence which is stably built into the chromosome can also be considerably increased. A suitable selection marker for it is the dihydrofolate-reductase (dhfr) for example. Mammal cells (for instance CHO cells), which contain no intact dhfr gene, will in that case be incubated with increasing amounts of methotrexate after completed transfection. In that way cell lines can be preserved that contain more than a thousand copies of the desired DNA sequence.

Mammal cells, which can be used for the expression, are for example cells of the human cell lines HeLa [ATCC CCL2] and 293 [ATCC CRL 1573], as well as 3T3- [ATCC CCL 163] and L cells, for instance [ATCC CCL 149], (CHO) cells [ATCC CCL 61], BHK [ATCC CCL 10] cells as well as the CV 1 [ATCC CCL 70] cell lines and the COS cell lines [ATCC CRL 1650, CRL 1651].

Suitable expression vectors include for example vectors like pBC12MI [ATCC 67 109], pSV2dhfr [ATCC 37 146], pSVL [Pharmacia, Uppsala, Sweden], pRSVCat [ATCC 37 152] and pMSG [Pharmacia, Uppsala, Sweden]. Particularly preferred vectors are the vectors "pK19" and "pN123" used in Example 9. These can be isolated from the E. coli strains HB101(pK19) and HB101(pN123), which are transformed with them, by means of known methods [42]. These E. coli strains were deposited on 26. January 1990 with the German Collection of Micro-Organisms and Cell Cultures, Inc. (DSM) in Braunschweig (BRD) [form. West Germany ..Transl.] under DSM 5761 for HB101(pK19) and DMS 5764 for HB101(pN123) .For the expression of proteins, which consist of a soluble fragment from non-soluble TNF-BP and an immunoglobulin component, i.e. all domains but the first from the constant region of the heavy chain, vectors derived from pSV2 like for example described by German, C. in "DNA Cloning" [Vol. II. edt by Glover, D.M., IRL Press Oxford, 1985], are particularly suitable. Particularly preferred vectors are the vectors pCD4-Hu (DSM 5315), pCD4-H 1 (DSM 5314) and pCD4-H 3 (DSM 5523), which are deposited with the German Collection of Micro-Organisms and Cell Cultures, Inc. (DSM) in Braunschweig, BRD and described in detail in the European Patent Application Nr. 90107393.2. The said European patent

specification mentioned, as well as the equivalent patent applications listed in Example 11, also contain material relating to the further use of these vectors for the expression of such chimeric proteins with other immunoglobulin components.

The nature and manner in which the cells are being transfected depends on the expression system and vector system chosen. A survey of these methods is found for example at Pollard et al. , "DNA Transformation of Mammalian Cells" in "Methods of Molecular Biology" [Nucleic Acids Vol.2, 1984, Walker, J.M., ed, Humana, Clifton, New Jersey]. Further methods are found at Chen and Okayama [" High-Efficiency Transformation of Mammalian Cells by Plasmid DNA", Molecular and Cell Biology 7, 2745-2752, 1987] and at Felgner [Felgner et al., "Lipofectin: a highly efficient lipid-mediated DNA-transfection procedure", Proc. Nat. Acad. Sci. USA 84, 7413-7417, 1987].

For the expression in insect cells the Baculovirus-Expression-System, which has already been employed successfully for the expression of a series of proteins, (for a summary see Luckow and Summers, Bio/Technology 6, 47-55, 1988), can be used. Recombinant proteins can be produced authentically or as fusion proteins. The proteins thus produced may also be modified, for example be glycosylated (Smith et al., Proc. Nat. Acad. Sci. USA 82, 8404-8408, 1987). To produce a recombinant Baculovirus, which expresses the desired protein, a so-called "transfer vector " is employed. This is understood to be a plasmid, which contains the heterologous DNA sequence under control of a strong promoter, for instance that of the polyhedrin gene, with the sequence being surrounded on both sides by viral sequences. Particularly preferred vectors are "pN113", "pN119" and "pN124", the vectors used in Example 10. These can be isolated according to known methods [42] from the E. coli strains HB101(pN113), HB101(pN119) and HB101(pN124). These E. coli strains were deposited with the German Collection of Micro-Organisms and Cell Cultures, Inc.

(DSM) in Braunschweig, BRD, under DSM 5762 for HB101(pN113), DSM 5763 for HB101(pN119) and DSM 5765 for HB101(pN124). The transfer vector is then transfected together with DNA from the wild type Baculovirus into the insect cells. The recombinant viruses that are generated in the cells through homologous recombination may then be identified and isolated according to known methods. A survey about the Baculovirus-Expression-System is found at Luckow and Summers [52].

Expressed TNF-BP as well as their non-soluble and soluble fragments can then be purified out through methods of the protein chemistry known at the current state of the technology, as for example the methods already described on pages 5-6, from the cell mass and or the culture supernatants.

The TNF-BP recovered according to the invention may also be used for the production of polyclonal and monoclonal antibodies according to known methods of the technology [44,45] or according to the method described in Example 3.

Such antibodies, particularly monoclonal antibodies against the 75 kDa TNF-BP species, are also a subject of the present invention. Such antibodies directed against the 75 kDa TNF-BP can be employed for isolation of TNF-BP by means of modifications familiar to the specialist to the purification method described in detail in Examples 4-6.

Based on the high binding affinity for TNF of the TNF-BP according to the invention (K_d values of the orders of magnitude of 10^{-9} - 10^{-10} M) they or fragments thereof may be used as diagnostics for identification of TNF in serum or in other body fluids according to methods known at the current state of technology, for instance in solid phase binding tests or in combination with anti-TNF-BP antibodies on so-called "sandwich" tests.

In general TNF-BP may be used according to the invention for purification of TNF on the one hand and for the location of TNF-agonists as well as TNF-antagonists by means of methods known at the current state of technology on the other.

The TNF-BP according to the invention as well as their physiologically compatible salts, which can be produced according to the methods known at the current state of technology, may also be used for production of pharmaceutical preparations in particular those for treatment of diseases in the course of which TNF is involved. One or several of the mentioned compounds, if desired resp. required in combination with other pharmaceutically active substances, may be processed in a known manner with the usually employed solid or liquid carrier materials. The dosage of such preparations can occur with reference to the usual criteria in analogy to preparations of similar activity and structure already in use.

After having described the above invention in general, the following examples are to illustrate details of the invention without limiting it by that in any way.

Example 1Detection of TNF-binding Proteins

The TNF-BP were detected in a filter test with human radioactive iodine ^{125}I -TNF. TNF (46,47) was marked radiactively with Na^{125}I (IMS40, Amersham, Amersham, England) and Iodo-Gen (#28600, Pierce Eurochemie, Oud-Beijerland, Netherlands) according to Fraker and Speck [48]. Isolated membranes of the cells or their solubilized, enriched and purified fractions, were applied to wetted cellulose nitrate filters (0.45 μ , Bio.Rad, Richmond, CA, USA) for detection of the TNF-BP. The filter was then blocked in buffer solution with 1% of defatted powdered milk and subsequently incubated, washed and airdried with $5 \cdot 10^5$ cpm/ml ^{125}I -TNF α ($0.3-1.0 \cdot 10^8$ cpm/ μg) in two batches with and without addition of 5 $\mu\text{g}/\text{ml}$ of unmarked TNF α . The bound radioactivity was detected through autoradiography semiquantitatively or counted in a γ -counter. The specific ^{125}I -TNF α binding was obtained after correction for nonspecific binding in the presence of unmarked TNF- α in the surplus. The specific TNF binding during the filter test was measured at various TNF concentrations and analyzed according to Scatchard [33], with a K_d value of $\sim 10^{-9} - 1 \cdot 10^{-10}$ having been obtained.

Example 2Cell Extracts from HL-60 Cells

HL60 cells [ATCC Nr. CCL 240] were cultivated on an experimental laboratory scale in an RPMI 1640-Medium [GIBCO catalog Nr. 074-01800], that also contained 2 g/l NaHCO_3 and 5% total calves' serum, within an atmosphere of 5% CO_2 and were subsequently centrifuged.

The following procedure was used to obtain high cell densities on a technical scale. Breeding was carried out in a 75 liter airlift fermenter (Chemap. Co. Swi-

tzerland) with 58 liter of working volume. For that purpose the cassette membrane system "PROSTAK" (Millipore, Switzerland) with a membrane surface of 0.32 m^2 (1 cassette) was integrated into the external circulation. The culture medium (see Table 1) was recirculated at 5 l/min. with a Watson-Marlow pump Type 603U. After sterilization of the equipment with steam, with the "PROSTAK" system being separately autoclaved, fermentation was started with growing HL-60 cells from a 20 l airlift fermenter (Chemap). Cell breeding in the seed fermenter was carried out in the medium through a conventional batch process according to Table 1 and an initial cell titre of 2×10^5 cells/ml. After 4 days the HL60 batch with a titre of 4.9×10^6 cells/ml was transferred to the 75 l fermenter. The pH value was kept at 7.1 and the pO_2 value at 25% saturation, with oxygen being fed through a micro-porous frit. After initial batch formation the perfusion was started on the second day with a cell titre of 4×10^6 cells/ml at a medium exchange rate of 30 l per day. On the filtrate side of the membrane the conditioned medium was withdrawn and replaced by inflow of fresh medium. The inflowing medium was strengthened as follows: primatone from 0.25% to 0.35%, glutamin from 5 mM to 6 mM and glucose from 4 g/l to 6 g/l. The perfusion rate was then raised on the third and fourth day to 72 l of medium/day and on the fifth day to 100 l of medium/day. After 120 hours of continuous breeding the fermentation was finished. Exponential cell growth up to 40×10^6 cells/ml occurred under the given conditions of fermentation. The period for doubling the cell population was 20-22 hours up to 10×10^6 cells/ml and increased to 30-36 hours with increasing cell density. The share of the living cells was around 90-95% during the entire period of fermentation. The HL-60 batch was then cooled down to about 12°C in the fermenter and the cells were recovered through centrifugation (Beckmann centrifuge [model J-6B, rotor JS], 3000 rpm, 10 min., 4°C).

Table 1

HL -60 Medium

Components	Concentrations mg/l
CaCl_2 (moisture free)	112,644
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$.20
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	$0.498 \cdot 10^{-3}$
$\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$	0.02
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.1668
KCl	336.72
KNO_3	0.0309
MgCl_2 (moisture free)	11.444
MgSO_4 (moisture free)	68.37
NaCl	5801.8
Na_2HPO_4 (moisture free)	188,408
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	75
$\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$	$9.6 \cdot 10^{-3}$
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.1726
D-Glucose	4000
Glutathion (red.)	0.2
Hepes-Buffer	2383.2
Hypoxanthin	0.954
Linoleic Acid	0.0168
Liponic Acid	0.042
Phenol Red	10.24
Putrescin 2HCl	0.0322
Na-Pyruvat	88
Thymidin	0.146

Biotin	0.04666
D-Ca-Pantothenate	2.546
Choline Chloride	5.792
Folic Acid	2.86
i-Inositol	11.32
Niacin Amide	2.6
para- Amino-Benzoic Acid	0.2
Pyridoxal HCL	2.4124
Pyridoxin HCL	0.2
Riboflavin	0.2876
Thiamin HCL	2.668
Vitamin B ₁₂	0.2782
L- Alanine	11.78
L- Asparagine Acid	10
L- Asparagine H ₂ O	14.362
L- Arginine	40
L- Arginine HCl	92.6
L- Aspartate	33.32
L- Cystine 2HCl	62.04
L- Cysteine HCl •H ₂ O	7.024
L- Glutamine Acid	36.94
L- Glutamine	730
L- Glycine	21.5
L- Histidine	3
L- Histidine HCl •H ₂ O	27.392
L- Hydroxypyroline	4
L- Isoleucine	73.788
L- Leucine	75.62

L- Lysine HCl	102.9
L- Methionine	21.896
L- Phenylalanine	43.592
L- Proline	26.9
L- Serine	31.3
L- Threonine	53
L- Tryptophan	11.008
L- Tyrosine •2Na	69.76
L- Valine	62.74
Penicillin/Streptomycin	100 U/ml
Insulin (human)	5 µg/ml
Transferrin (human)	15 µg/ml
Bovine Serum Albumin	67 µg/ml
Primatone RL (Sheffield Products, Norwich, N.Y., USA)	0.25%
Pluronic F68 (Serva, Heidelberg, BRD)	0.01%
Fetal Calves' Serum	0.3-3%

The centrifugate was washed with isotonic phosphate buffer (PBS; 0,2g/l KCl, 0.2 g/l KH_2PO_4 , 8.0 g/l NaCl, 2.16 g/l $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$), to which were added 5% dimethyl formamide, 10 mM benzamidine, 100 E/ml aprotinine, 10 µM leupeptine, 1 µM pepstatine, 1 mM o-pheanthroline, 5 mM iodacetamide, 1mM phenylmethylsulphonyl fluoride (and which will be referred to as PBS-M from now on). The washed cells were extracted at a cell density of $2.5 \cdot 10^8$ cells/ml in PBS-M with Triton X-100 (final concentration 1.0%). The cell extract was clarified through centrifugation (15'000 x g, 1 hour; 100'000 x g, 1 hour).

Example 3Production of monoclonal (TNF-BP) Antibodies

A supernatant of centrifugation obtained according to Example 2 from cultivation of HL60 cells on an experimental laboratory scale was diluted 1:10 with PBS. The diluted supernatant was applied at 4°C to a column (flow rate: 0.2 ml/min), which contained 2 ml Affigel 10 (Bio Rad catalog Nr. 153-6099), to which 20 mg of recombinant human TNF- α [Pennica, D. et al. (1984) *Nature* 312, 724; Shirai, T. et al. (1985) *Nature* 313, 803; Wang, A.M. et al. (1985) *Science* 228, 149] were coupled according to the suggestions of the manufacturer. The column was washed at 4°C and a flow-through rate of 1 ml/min first with 20 ml of PBS containing 0.1% Triton X 114 and then with 20 ml PBS. The thus enriched TNF-BP was eluted at 22°C and a flow rate of 2 ml/min with 4 ml of 100 mM glycine, pH 2.8, 0.1% decylmaltoside. The eluate was concentrated to 10 μ l in a Centricon 30 unit [Amicon].

10 μ l of this eluate were mixed with 20 μ l of complete Freund's adjuvant to an emulsion. According to the procedure described by Holmdahl, R. et al. [(1985), *J. Immunol. Methods* 83 379] 10 μ l of the emulsion were injected on days 0, 7 and 12 into a rear paw of an anesthetized Balb/c mouse.

On day 14 the immunized mouse was killed, the popliteal lymph node removed, chopped up and suspended in Iscove's medium (MEM, GIBCO catalog Nr. 074-2200), which contained 2 g/l NaHCO₃, through repeated pipetting. According to a modified procedure by De St. Groth and Scheidegger [*J. Immunol. Methods* (1980), 35, 1] 5×10^7 cells of the lymph node were fused with 5×10^7 PAI mouse myeloma cells (J.W. Stocker et al., *Research Disclosure*, 217, May 1982, 155-157), which were in the process of logarithmic growth. The cells were mixed, collected through centrifugation then resuspended through gentle shaking in 2 ml 50% (v/v) polyethylene glycol in IMEM ~~at room temperature and diluted through slow addition of 10 ml IMEM during 10 minutes of careful shaking.~~ The cells were collected through centrifugation and re-

suspended in 200 ml of the complete medium [IMEM + 20% fetal calves' serum, glutamine (2.0 mM), 2-mercaptoethanol (100 μ M), 100 μ M hypoxanthine, 0.4 μ M aminopterin and 16 μ M thymidine (HAT)]. The suspension was distributed among 10 tissue culture dishes, each with 96 indentations and ,without change of medium, incubated at 37°C in an atmosphere of 5% CO₂ and at relative humidity of 98%,for 11 days.

The antibodies are characterized by their inhibiting effect to TNF binding with HL60 cells or through their binding to antigen during the filter test according to Example 1. The following procedure was employed for detection of biological activity of anti-(TNF-BP)-antibodies : 5x10⁶ HL60 or U937 cells were incubated in the complete RPMI 1640 medium together with affinity-free monoclonal anti-(TNF-BP) antibodies or control antibodies (i.e. those that are not directed against TNF-BP) in a concentration range of 1 ng/ml to 10 μ g/ml. After an hour of incubation at 37°C the cells were collected through centrifugation and washed with 4.5 ml PBS at 0°C . They were resuspended in 1 ml of complete RPMI 1640 medium (Example 2), which contained additional 0.1% sodium azide and ¹²⁵I-TNF α (10⁶ cpm/ml) with or without addition of unmarked TNF α (see above). The specific radioactivity of ¹²⁵I- TNF α amounted to 700 Ci/mmol. The cells were incubated for 2 hours at 4°C, collected and washed four times with 4.5 ml PBS, which contained 1% BSA and 0.001% Triton X 100 (Fluka), at 0°C. The radioactivity linked to the cells was measured in a γ - scintillation counter . In a comparable experiment the radioactivity linked to cells that were not treated with anti-(TNF-BP) antibodies was determined (about 10,000 cpm/5x10⁶ cells).

Example 4

Affinity Chromatography

For further purification a monoclonal anti-(44 kDa TNF-BP) antibody (2.8 mg/ml gel),as obtained according to Example 3, TNF (3.0 mg/ml gel) and bovine serum albumin (BSA, 8.5 mg/ml gel)were coupled covalent to CNBr-activated Sepharose 4B

(Pharmacia, Uppsala, Sweden). The cell extract obtained according to Example 2 was fed through the thus established columns which were arranged in series in the following sequence: BSA-Sepharose precolumn, immunoaffinity column [anti-(55 kDa-TNF-BP)-antibody], TNF α -ligand affinity column. After the completed task the two last mentioned columns were separated and individually washed with 100 ml each of the following buffer solutions: (1) PBS, 1.0% Triton X-100, 10 mM benzamidine, 100 E/ml aprotinine; (2) PBS, 0.1% Triton X-100, 0.5 M NaCl, 10 mM benzamidine, 100 E/ml aprotinine; and (3) PBS, 0.1% Triton X-100, 10mM benzamidine, 100 E/ml aprotinine. The immunoaffinity column as well as the TNF α -ligandaffinity column were then eluated, each one separately, with 100 mM glycine pH 2.5, 100 mM NaCl, 0.2% decylmaltoside, 10 mM benzamidine, 100 E/ml aprotinine. The fractions of each column that were active during the filter test as per Example 1 were always united afterwards and neutralized with 1 M Tris pH 8.0 .

The united TNF-BP active fractions of immunoaffinity chromatography on the one hand and of TNF α - ligandaffinity chromatography on the other were once more applied each to a small TNF α - ligandaffinity column for further purification. Afterwards these two columns were washed each with 40 ml of (1) PBS, 1.0% Triton X-100, 10 mM benzamidine, 100 E/ml aprotinine, (2) PBS, 0.1% Triton X-100, 0.5 M NaCl, 10 mM ATP, 10 mM benzamidine, 100 E/ml aprotinine, (3) PBS, 0.1% Triton X-100, (4) 50 mM Tris pH 7.5, 150 mM NaCl, 1.0% NP-40, 1.0% desoxycholate, 0.1% SDS, (5) PBS, 0.2% decymatoside .Subsequently the columns were eluated with 100 mM glycine pH 2.5, 100 mM NaCl, 0,2% decylmaltoside. Fractions of 0.5 ml from each column were collected separately and the active fractions per filter test (Example 1) of each column united each separately and concentrated in a Centricon unit (Amicon, Molecular weight exclusion 10'000).

Example 5

Separation by means of HPLC

The active fractions obtained according to Example 4 were applied with reference to the different origins (immunoaffinity chromatography resp. ligandaffinity chromatography) each separately to C1/C8 reversed-phase HPLC columns (ProRPC, Pharmacia, 5x20 mm), which had been equilibrated with 0.1% trifluoroacetic acid, 0.1% octylglucoside. The columns were then eluted with a linear acetonitrile gradient (0-80%) in the same buffer with a flow of 0.5 ml/min. Fractions of 1.0 ml were collected from each column and the active fractions from each column were separately united (detection per Example 1).

Example 6

Separation by means of SDS-PAGE

The active fractions obtained according to Example 5 and per filter test (Example 1) were further separated according to [34]. For that purpose the probes were heated for three minutes to 95°C in SDS probe buffer and subsequently separated electrophoretically on a 12% acrylamide separation gel with a 5% collecting gel. As reference for determination of the apparent molecular masses on the SDS-PAGE gel the following reference proteins were used: phosphorylase B (97.4 kDa), BSA (66.2 kDa), ovalbumin (42.7 kDa), Carboanhydrase (31.0 kDa), soy trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

Under the conditions mentioned two bands of 55 kDa and 51 kDa as well as three weaker bands of 38 kDa, 36 kDa and 34 kDa were obtained for probes that had been obtained according to Example 4 through TNF α -ligandaffinity chromatography from immunoaffinity chromatography eluates and had been further separated through HPLC according to Example 5. These bands were transferred electrophoretically in a Mini Trans Blot System (Bio Rad, Richmond, CA, USA) for 1 hour at 100 V in 25 mM Tris, 192 mM glycine, 20% methanol to a PVDF membrane (Immobilon, Millipore, Bedford, Mass. USA). Afterwards the PVDF membrane was dyed either with 0.15% Serva-Blau (Serva, Heidelberg, BRD) in methanol/water/glacial acetic acid (50/40/10 parts by volume) to protein or blocked with defatted powdered milk and subse-

quently incubated with ^{125}I -TNF α according to the filter test conditions described in Example 1 for the detection of bands with TNF-BP activity. It was shown that all the bands exhibited in the protein color specifically bound TNF α . All these bands bound in the Western blot according to Towbin et al. [38] including the monoclonal anti-55 kDa-TNF-BP- antibody produced according to Example 3. In that case a rabbit-anti-mouse -immunoglobulin antibody marked by Na ^{125}I radioactively, affinity-free(mouse immunoglobulin -Sepharose-4B affinity column) according to the process described in Example 1 was employed for autoradiographic detection of this antibody.

Probes, which are obtained according to Example 4 through double TNF α - ligandaffinity chromatography of the flowthrough of immunoaffinity chromatography and are further separated through HPLC according to Example 5, showed two additional bands of 75 kDa and 65 kDa, both of which bound TNF specifically during the filter test (Example 1). In the Western blot according to Towbin et al. (see above) the proteins of these two bands did not react with the anti-(55 kDa TNF-BP)- antibody produced according to Example 3. They did react, however, with a monoclonal antibody, which had been produced according to Example 3 starting from the 75 kDa band (anti-75 kDa TNF-BP-antibody).

Example 7

Amino Acid Sequence Analysis

For amino acid sequence analysis the active fractions obtained according to Example 5 and per filter test (Example 1) were separated by means of the SDS-PAGE conditions described in Example 6 and now being reduced (SDS probe buffer with 125 mM dithiothreitol). The same bands were found as per Example 6, but they all showed about 1-2 kDa higher molecular masses in comparison with Example 6 because of the reducing conditions of SDS-PAGE. These bands were then transferred according to Example 6 to PVDF membranes and dyed with 0.15% 35 Serva-Blau in methanol/water/glacial acetic acid (50/40/10 parts per volume) during 1 minute,

decolored with methanol/water/glacial acetic acid (45/48/7 parts by volume), rinsed with water, airdried and then cut out. During all the steps the conditions given by Hunkapiller [34] were followed to avoid N-terminal blockage. At first the purified TNF-BP were placed unchanged into the amino acid sequencing. To obtain additional sequence information, the TNF-BP were cleaved with bromocyanide (Tarr, G.E. in "Methods of Protein Microcharacterization", 165-166 op. cit.), trypsin and/or proteinase K, after reduction and S-carboxymethylation [Jones, B.N. (1986) in "Methods of Microcharacterization", J.E. Shively, ed. Human Press, Clifton N.J. 124-125] and the peptides were separated by means of HPLC according to known methods of protein chemistry. So prepared the probes were then sequenced in an automated gas phase-microsequencing apparatus (Applied Biosystems Model 470A, ABI, Foster City, CA, USA) with an on-line automated HPLC PTH- amino acid analyzer (Applied Biosystems Model 120, ABI see above) behind it, with the following amino acid sequences being determined :

1. For the 55 kDa band (according to non-reducing SDS-PAGE):

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile

and

Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys

where X stands for an amino acid residue that could not be identified.

2. For the 51 kDa and the 38 kDa bands (according to non-reducing SDS-PAGE):

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu

3. For the 65 kDa band (according to non-reducing SDS-PAGE): during N-terminal sequencing of the 65 kDa band two parallel sequences were identified without interruption down to the 15th residue. Since one of the sequences corresponded to a partial sequence of ubiquitin [36,37] the following sequence was derived for the 65 kDa band:

Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys.

where X stands for an amino acid residue that could not be identified.

Additional peptide sequences for 75(65)kDa-TNF-BP were identified:

Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu

and

Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-LeuLeu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu

and

Val-Phe-Cys-Thr

and

Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala

and

Leu-Cys-Ala-Pro

and

Val-Pro-His-Leu-Pro-Ala-Asp

and

Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro

Where X stands for an amino acid residue that could not be identified.

Example 8

Analysis of Base Sequences of Complementary DNA (cDNA)

Starting with the amino acid sequence according to formula AI, complete degenerated oligonucleotides corresponding to amino acid residues 2-7 and 17-23 were, with consideration of the genetic code, synthesized in suitable complementarity ("sense" and "antisense" oligonucleotide). Total cellular RNA was isolated from HL60 cells [42,43], and the first strand of cDNA was synthesized through oligo-dT-priming or through priming with the "antisense" oligonucleotide by means of a cDNA synthesis kit (RPN 1256 Amersham, Amersham England) according to the manufacturer's instructions. This cDNA strand and the two synthesized degenerated "sense" and "antisense" oligonucleotides were used in a polymerase chain reaction

(PCR, Perkin Elmer Cetus, Norwalk, CT, USA, according to manufacturer's instruction) to synthesize the base sequence encoded for the amino acid residues 8-16 (formula IA) as a cDNA fragment. The base sequence of this cDNA fragment is as follows: 5'-AGGGAGAAGAGAGATAGTGTGTGTCCC-3'. This cDNA fragment was used as probe to identify a cDNA clone encoded for the 55 kDa TNF-BP in a λ gt 11-cDNA-gene bank of human placenta (42,43) through known procedures. This clone was then cut from the λ -vector with the usual methods and cloned into the M13mp18/M13mp19 bacteriophages (Pharmacia, Uppsala, Sweden) (42,43). The nucleotide sequence of this cDNA clone was determined by means of a sequenase kit (U.S. Biochemical, Cleveland, Ohio, USA) according to information from the manufacturer. The nucleotide sequence and derived from it the amino acid sequence for the 55 kDa TNF-BP and its signal peptide (amino acid "-28" to amino acid "0") is illustrated in Fig.1 with abbreviations for bases and amino acids customary at the present state of technology. From sequence comparisons with other already known receptor protein sequences about 180 N-terminals containing amino acids as well as 220 C-terminal domains containing amino acid may be identified, which are separated by a transmembrane region of 19 amino acids (underlined in Fig.1) typical according to sequence comparisons. Hypothetical glycosylation locations are marked with stars above the corresponding amino acid in Fig.1 .

Analog technologies were employed basically for identification of partial cDNA sequences encoded for 75/65 kDa TNF-BP with, in this case, genomic human DNA and from peptide IIA derived completely degenerated 14-mer (polymer ..Transl.) and 15-mer "sense and "antisense" nucleotides being used, to produce a primary, 26 bp cDNA probe in a polymerase chain reaction. This cDNA probe was then used for identification of cDNA clones of various lengths in a HL-60 CDNA library. This cDNA library was produced by means of isolated HL60 RNA and a cDNA cloning kit (Amersham) per instructions of the manufacturer. The sequence of such a cDNA clone is illustrated in Fig.4, with repeated sequencing leading to the

following correction . In place of the serine in position 3 a threonine must be put which is encoded by "ACC" and not by "TCC".

Example 9

Expression in COS 1-Cells

For the expression in COS cells vectors were constructed which originated from plasmid "pN11". The plasmid "pN11" contains the efficient promoter and enhancer of the "major immediate-early " gene of the human cytomegalovirus ("HCMV"; Boshart et al., Cell 41 , 521-530, 1985). Behind the promoter there is a short DNA sequence, containing several restriction interfaces that occur only once in the plasmid ("polylinker"), among them the interfaces for HindIII, Ball, BamHI and PvuII (see sequence).

PvuII

5'- AAGCTTGGCCAGGATCCAGCTGACTGACTGATCGCGAGATC-3'

3'- TTCGAACCGGTCCTAGGTCGACTGACTGACTAGCGCTCTAG-5'

Behind these interfaces there are three translation stop codons in all three reading rasters . Behind the polylinker sequence is the second intron and the polyadenylation signal for the preproinsulin gene of the rat (Lomedico et al. Cell 18, 545-558, 1979). The plasmid contains furthermore the replication origin of the SV40 virus as well as a fragment from pBR322, which confers ampicillin resistance on E. coli bacteria and makes possible the replication of the plasmid in E. coli .

For construction of the expression vector "pN123" this plasmid "pN11" was cut with the restriction endonuclease PvuII and subsequently treated with alkaline phosphatase. The dephosphorylated vector was then isolated from an agarose gel (V1). The 5' overhanging nucleotides of the EcoRI cut 1.3 kb fragments of the 55 kDa TNF-BP=cDNA (see Example 8) were filled with the help of Klenow enzyme. Subsequently this fragment was isolated from an agarose gel (F1).

Afterwards V1 and F1 were united by means of T4 ligase. *E. coli* HB 101 cells were then transformed with this ligation batch according to familiar methods [42]. With the aid of restriction analyses and DNS sequencing according to familiar methods [42] transformants were identified, which had been transformed with a plasmid that contained the 1.3kb EcoRI fragment of the 55 kDa TNF-BP-cDNA in correct orientation for the expression via the HCMV promoter. This vector received the designation "pN123".

The following procedure was used for construction of the vector "pK19". A DNA fragment, which contains only the cDNA coded for the extracellular part of the 55 kDa TNF-BP (amino acids -28 to 182 according to Fig.1) was obtained by means of PCR technology (Saiki et al., Science 230, 1350-1354, 1985, see also Example 8). The following oligonucleotides were used for amplification of the cDNA encoded for the extracellular part of 55 kDa TNF-BP from "pN123".

BAMHI

5'-CACAGGGATCCATAGCTGTCTGGCATGGGCTCTCCAC-3'

ASP718

3'-CGTGACTCCTGAGTCCGTGGTGTATTATCTCTAGACCATGGCCC-5'

By means of these nucleotides two stop codons of the translation were also introduced behind amino acid 182. The thus amplified fragment was cut with BamHI and Asp718 and the resulting projecting ends were filled with the aid of the Klenow enzyme; this fragment was subsequently isolated from an agarose gel (F2). F2 was then ligated with V1 and the whole batch used for transformation of *E. coli* HB101, as already described. Transformants which were transformed with a plasmid that contained the DNA fragment in correct orientation for expression via the HCMV-promoter, were identified by means of DNA sequencing (see above). The plasmid isolated from it received the designation "pK19".

~~Transfection of the COS cells with plasmids "pN123 or "pK19" was carried~~
out according to the lipofection method published by Felgner et al. (Proc. Natl.

Acad. Sci. USA 84 , 7413-7417, 1987). 72 hours after the completed transfection the cells transfected with "pN123" were analyzed according to known methods with ^{125}I -TNF α for binding. The result of the Scatchard analysis [Scatchard, G., Ann. N.Y. Acad. Sci. 51, 660, 1949] of binding data obtained in that way (Fig.2A) is illustrated in Fig. 2B. The culture supernatants of the cells transfected with "pK19" were investigated in a "sandwich" test. For that purpose PVC microtitre plates (Dynatech, Arlington, VA, USA) with 100 μ /hole of a rabbit-anti-mouse immunoglobulin (10 μ g/ml PBS) were sensitized. The plate was subsequently washed and incubated (3 hours, 20°C) with an anti-55 kDa TNF-BP antibody, which was detected according to Example 3 through its antigen binding and isolated, but does not inhibit TNF binding to cells. The plate was then washed again and incubated overnight at 4°C with 100 μ l/hole of the culture supernatants (diluted 1:4 with buffer A containing 1% defatted milk powder : 50 mM Tris/HCl pH 7.4, 140 mM NaCl, 5 mM EDTA, 0.02% Na-azide). The plate was emptied and incubated with buffer A (10⁶ cpm/ml, 100 μ l/hole) containing ^{125}I -TNF α with or without addition of 2 μ g/ml unmarked TNF for two hours at 4°C . Afterwards the plate was washed four times with PBS, the individual holes were cut out and measured in a γ -counter. The results of five parallel transfections (columns # 2,3,4,6 and 7), of two control transfections with the pN11 vector (columns #1,5) and of a control with HL60-cell lysate (column #8) are illustrated in Fig. 3.

Example 10

Expression in Insect Cells

For expression in a baculovirus expression system a start was made with the plasmid "pVL941" (Luckow and Sommers, 1989, "High Level Expression of Nonfused Foreign Genes with Autographa California Nuclear Polyhedrosis virus Expression Vectors", Virology 170, 31-39), which was modified as follows. The sole EcoRI-interface in "pVL941" was removed by cutting the plasmid with EcoRI and filling

the protruding 5' ends with Klenow enzyme. The thus obtained plasmid pVL941/E- was digested with BamHI and Asp718 and the vector rump subsequently isolated from an agarose gel. This fragment was ligated with a synthetic oligonucleotide of the following sequence:

	BamHI	EcoRI	Asp718	
5'	-	GATCCAGAATTCATAATAG	-	3'
3'	-	GTCTTAAGTATTATCCATG	-	5'

E. coli HB101 was transformed with the ligation batch and transformants, which contained a plasmid in which the oligonucleotide was correctly inserted, were identified through restriction analysis and DNA sequencing according to known methods (see above); this plasmid was called "PNR704". For construction of the transfer vector "pN113" this plasmid "pNR704" was cut with EcoRI, treated with alkaline phosphatase and the thus generated vector rump (V2) was subsequently isolated from an agarose gel. The 1.3 kb fragment of 55 kDa TNF-BP-cDNA, cut with EcoRI like above, was ligated with fragment V2. Transformants obtained with this ligation batch, which contained a plasmid that contained the cDNA insert in the correct orientation for expression via the polyhedrin promoter, were identified (see above). The vector isolated from it received the designation "PN113".

The following steps were taken for construction of the transfer vector "pN119". The 1.3 kb EcoRI/EcoRI-fragment of the 55 kDa TNF-BP cDNA in the "pUC19" plasmid (see Example 8) was digested with BanI and ligated with the following synthetic oligonucleotide :

	BanI	Asp718
5'	-	GCACCACATAATAGAGATCTGGTACCGGGAA - 3'
3'	-	GTGTATTATCTCTCTAGACCATGGCCC - 5'

By means of the above adapter two stop codons of the translation behind amino acid 182 and an interface for the restrictions endonuclease Asp718 are

inserted. After completed ligation the batch was digested with EcoRI and Asp718 and the partial 55 kDa TNF-BP fragment (F3) was isolated. Furthermore plasmid "pNR704", which was also cut with Asp718 and EcoRI, was ligated with F3 and the ligation batch transformed in *E. coli* HB101. Identification of the transformants, which contained a plasmid in which the partial 55 kDa TNF-BP cDNA was integrated correctly for the expression, was carried out as already described. The plasmid isolated from these transformants received the name "pN119".

The following procedure was followed in construction of the transfer vector "pN124". The cDNA fragment described in Example 9, which encodes for the extra-cellular part of 55 kDa TNF-BP, was amplified with the oligonucleotides mentioned by means of PCR technology, as described in Example 9. This fragment was cut with BamHI and Asp718 and isolated from an agarose gel (F4). The plasmid "pNR704" was also cut with BamHI and Asp718 and the vector rump (V4) was isolated (see above). The fragments V4 and F4 were ligated, used to transform *E. coli* HB101 and the recombinant transfer vector "pN124" was identified and isolated, as described.

The following procedure was used for transfection of insect cells. 3 µg of the transfer vector "pN113" were transfected with 1 µg DNA of Autographa californica-nuclear-polyhedrosis virus (AcMNPV) (EP 127839) in Sf9 cells (ATCC CRL 1711). Polyhedrin negative viruses were identified and purified from "Plaques" [52]. With these recombinant viruses Sf9 cells were again infected as described in [52]. After 3 days in the culture the infected cells were examined for binding of TNF with ^{125}I -TNF α . For that purpose the transfected cells were washed with a Pasteur pipette from the cell culture dish and, with a cell density of 5×10^6 cells /ml culture medium [52] containing 10 ng/ml ^{125}I -TNF α , as well as in the presense and absence of 5 µg/ml unmarked TNF α , they were resuspended and incubated on ice for two hours. Afterwards the cells were washed with a clean culture medium and the cellbound radioactivity was counted with a γ -counter (see

Table 2].

Table 2

Cells	Cellbound radioactivity/10 ⁶ cells
Non-infected cells (control)	60 cpm
Infected cells	1600 \pm 330 cpm ¹⁾

¹⁾ Average value and std. deviation from 4 experiments

Example 11

Analogous to the method described in Example 9 the cDNA fragment encoded for the extracellular range of the 55 kDa TNF-BP was now amplified in a polymerase chain reaction with the following oligonucleotides as primers :

Oligonucleotide 1 :

Sst I

5' - TAC GAG CTC GGC CAT AGC TGT CTG GCA TG - 3'

Oligonucleotide 2 :

Sst I

5' - ATA GAG CTC TGT GGT GCC TGA GTC CTC AG -3'

This cDNA fragment was ligated into the pCD4-Hy 3-vector [DSM 5523; European Patent Application Nr. 90107393.2; Japanese Patent Application Nr. 108967/90; US Patent Application Ser. No. 510773/90], from which the CF4-cDNA had been removed via the Sst I-restriction interfaces. Sst interfaces are present in vector pCD4-H 3 before, inside and behind the CD4 partial sequence section. The construct was transfected by means of protoplast fusion according to Of et al. (Proc. Nat. Acad. Sci. USA 80 , 825-829, 1983) in J558-myeloma cells (ATCC Nr. TIB6). Transfectants were

selected into the basic medium (Dulbecco's modified Eagle's medium, 10% fetal calves' serum, 5×10^{-5} M 2-mercaptoethanol) by addition of 5 $\mu\text{g/ml}$ myphenolic acid and 250 $\mu\text{g/ml}$ xanthine (Traunecker et al., Eur. J. Immunol. 16, 851-854 [1986]). The expression product secreted by the transfected cells could be purified through the usual methods of protein chemistry, for instance TNF-BP antibody affinitychromatography. Where not already specifically mentioned, standard methods like for example that of Freshney, R.I. in "Culture of Animal Cells", Alan R. Liss, Inc. New York (1983) were used for growing the cell lines used, for cloning, selecting, resp. for expansion of the cloned cells.

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Claims

1. Non-soluble proteins and soluble or non-soluble fragments thereof, which bind TNF in homogeneous form, as well as their physiologically compatible salts.
2. Compounds according to Claim 1, which are characterized by molecular masses according to SDS-PAGE under nonreducing conditions of about 55 kDa and 75 kDa.
3. Compounds according to one of the Claims 1 and 2, which contain at least one

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-
 Ile-His-Pro-Gln-X-Asn-Ser-Ile;
 Ser-Thr-Pro-Glu-Lys-Glu-GlyGlu-Leu-Glu-Gly-Thr-Thr-Thr-Lys;
 Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys;
 Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Glu-Pro-Leu-Glu;
 Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu;
 Val-Phe-Cys-Thr;
 Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala;
 Leu-Cys-Ala-Pro;
 Val-Pro-His-Leu-Pro-Ala-Asp;
 Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro

where X stands for an unidentified acid residue.

4. TNF sequences which are encoded for non-soluble proteins or soluble as well as non-soluble fragments thereof, that bind TNF, with such DNS sequences to be selected from the following:

(a) DNA sequences, as shown in Fig.1 or Fig.4, as well as their complementary strands, or those that these sequences include;

(b) DNA sequences, which hybridize with sequences as defined under (a) or with their fragments.

(c) DNA sequences, which do not hybridize with sequences as defined under (a) and (b), because of degeneracy of the genetic code, but which are encoded for polypeptides with exactly the same amino acid sequence.

5. DNA sequences according to Claim 4, which include a combination of two partial DNA sequences, with one partial sequence encoded for soluble fragments of non-soluble proteins that bind TNF, and the other partial sequence encoded for all domains save the first of the constant region of the heavy chain of human immunoglobulins, like IgG, IgA, IgM resp. IgE.

6. DNA sequences according to Claim 5, with said human immunoglobulins being of

the IgM type resp. those of class IgG.

7. DNA sequences according to Claim 6, where said human immunoglobulins are those of type Ig1 resp. Ig3.

8. Recombinant proteins from DNA sequences encoded according to one of the Claims 4-7, such as allelic variants, or deletion analogs, substitution analogs or addition analogs thereof.

9. Vectors, which contain DNA sequences according to one of the Claims 4-7 and are suitable for expression of proteins encoded by these DNA sequences in prokaryotic as well as eukaryotic host systems.

10. Prokaryotic as well as eukaryotic host systems which have been transformed with a vector according to Claim 9.

11. Host systems according to Claim 10, which are mammalian or insect cells.

12. Antibodies directed against a compound according to one of the Claims 1-3 or 8.

13. A method for isolation of a compound according to one of the Claims 1-3, characterized in that the following purification steps are carried out sequentially: production of a cell extract, immunoaffinity chromatography and/or simple or multiple ligandaffinity chromatography, HPLC and preparative SDS-PAGE and if so desired cleavage of the compounds thus isolated and/or transference into suitable salts.

14. A method for production of a compound per Claim 8, which is characterized in that a transformed host system according to Claim 10 or 11 is cultivated in a suitable medium and in that such compounds are isolated from the host system proper or from the medium.

15. Pharmaceutical preparations, characterized in that they contain one or more compounds according to one of the Claims 1-3 or 8, if so desired in combination with additional pharmaceutically effective substances and/or non-toxic, inert, therapeutically compatible carrier materials.

16. Pharmaceutical preparations for treatment of diseases, in which TNF is involved, where such preparations are characterized in that they contain one or more compounds

according to one of the Claims 1-3 or 8, if so desired in combination with additional pharmaceutically effective substances and/or non-toxic, inert, therapeutically compatible carrier materials.

17. Use of a compound according to one of the Claims 1-3 or 8 for treatment of diseases.

18. Use of a compound according to one of the Claims 1-3 or 8 for treatment of diseases, in which TNF is involved.

19. A compound as claimed in one of the Claims 1-3 or 8 whenever it has been produced by a method as claimed in Claims 13 or 14.

Figur 1

Fig.1

-185 GAATTCGGGGGGGTTCAAGATCACTGGGACCAGGCCGTGATCTCTATGCCCGAGTCTCAA
 -125 CCCTCAACTGTCACCCCAAGGCACTTGGGACGTCCTGGACAGACCGAGTCCCGGGAAGCC
 -65 CCAGCACTGCCGCTGCCACACTGCCCTGAGCCCAATGGGGGAGTGAGAGGCCATAGCTG
 -28.
 -30 MetGlyLeuSerThrValProAspLeuLeuLeuProLeuValLeuLeuGluLeu
 -5 TCTGGCATGGGCCTCTCCACGTCCTGACCTGCTGCTGCCGCTGGTGCTCCTGGAGCTG
 +1
 -10 LeuValGlyIleTyrProSerGlyValIleGlyLeuValProHisLeuGlyAspArgGlu
 55 TTGGTGGGAATATACCCCTCAGGGGTATTGGACTGGTCCCTCACCTAGGGGACAGGGAG

 10 LysArgAspSerValCysProGlnGlyLysTyrIleHisProGlnAsnSerIleCys
 115 AAGAGAGATAGTGTGTGTCCCAAGGAAATATATCCACCCTCAAATAATTGATTTC
 30 CysThrLysCysHisLysGlyThrTyrLeuTyrAsnAspCysProGlyProGlyGlnAsp
 175 TGTACCAAGTGCCACAAAGGAACCTACTTGTACAATGACTGTCCAGGCCCGGGGACAGGAT
 50 ThrAspCysArgGluCysGluSerGlySerPheThrAlaSerGluAsnHisLeuArgHis
 235 ACGGACTGCAGGGAGTGTGAGAGCGGCTCCTTCACCGCTCAGAAAACCCACCTCAGACAC
 70 CysLeuSerCysSerLysCysArgLysGluMetGlyGlnValGluIleSerSerCysThr
 295 TGCCTCAGCTGCTCCAAATGCCGAAAGGAAATGGGTGAGGTGGAGATCTCTTCTTGACA
 90 ValAspArgAspThrValCysGlyCysArgLysAsnGlnTyrArgHisTyrTrpSerGlu
 355 GTGGACCGGGACACCGTGTGTGGCTGCAGGAAGAACCTGACCGGCATTATTGGAGTGAA

 110 AsnLeuPheGlnCysPheAsnCysSerLeuCysLeuAsnGlyThrValHisLeuSerCys
 415 AACCTTTTCCAGTGCTTCAATTGCAGCCTCTGCTCAATGGGACCGTGCACCTCTCCTGC
 130 GlnGluLysGlnAsnThrValCysThrCysHisAlaGlyPhePheLeuArgGluAsnGlu
 475 CAGGAGAAACAGAACACCGTGTGCACCTGCCATGCAGGTTTCTTTCTAAGAGAAAACGAG
 150 CysValSerCysSerAsnCysLysLysSerLeuGluCysThrLysLeuCysLeuProGln
 535 TGTGTCTCCTGTAGTAAGTGTAAAGAAAAGCCTGGAGTGCACGAAGTTGTGCCTACCCAG
 170 IleGluAsnValLysGlyThrGluAspSerGlyThrThrValLeuLeuProLeuValIle
 595 ATTGAGAAATGTTAAGGGCACTGAGGACTCAGGCACCACAGTGTGTGCCCCCTGGTCATT
 190 PhePheGlyLeuCysLeuLeuSerLeuLeuPheIleGlyLeuMetTyrArgTyrGlnArg
 655 TTCTTTGGTCTTTGCCTTTTATCCCTCCTCTTCATTGGTTTAATGTATCGCTACCAACGG
 210 TrpLysSerLysLeuTyrSerIleValCysGlyLysSerThrProGluLysGluGlyGlu
 715 TGGAGTCCAAGCTCTACTCCATTGTTTGTGGGAAATCGACACCTGAAAAGAGGGGGAG

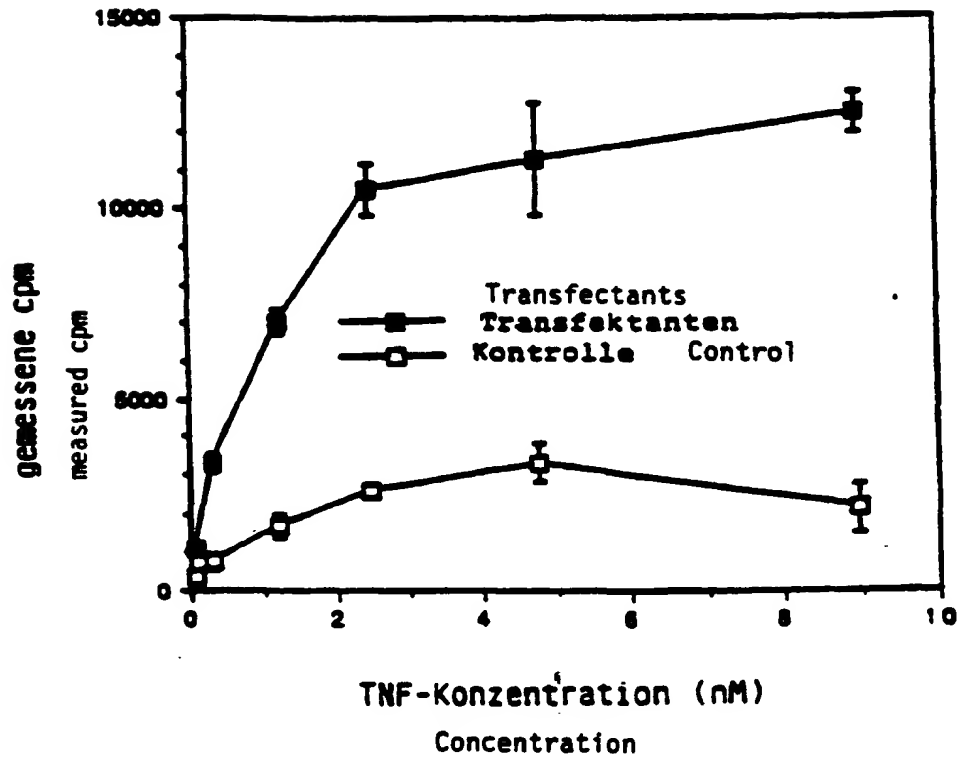
 230 LeuGluGlyThrThrThrLysProLeuAlaProAsnProSerPheSerProThrProGly
 775 CTGAAGGAATACTACTAAGCCCTGGCCCCAAACCAAGCTTCAGTCCCACTCCAGGC
 250 PheThrProThrLeuGlyPheSerProValProSerSerThrPheThrSerSerSerThr
 835 TTCACCCCAACCTGGGCTTCAGTCCCGTGCCAGTTCACCTTCACCTCCAGCTCCACC
 270 TyrThrProGlyAspCysProAsnPheAlaAlaProArgArgGluValAlaProProTyr
 895 TATACCCCGGTGACTGTCCCACTTTGCGGCTCCCGCAGAGAGGTGGCACCACCTAT
 290 GlnGlyAlaAspProIleLeuAlaThrAlaLeuAlaSerAspProIleProAsnProLeu
 955 CAGGGGGCTGACCCCATCCTTGGCAGACCCCTCGCCTCCGACCCCATCCCAACCCCTT

Figur 1 (Forts.) Fig.1 (cont.)

310 GlnLysTrpGluAspSerAlaHisLysProGlnSerLeuAspThrAspAspProAlaThr
 1015 CAGAAGTGGGAGGACAGCGCCCAAGCCACAGAGCCTAGACACTGATGACCCCGCGACG
 330 LeuTyrAlaValValGluAsnValProProLeuArgTrpLysGluPheValArgArgLeu
 1075 CTGTACGCCCGTGGTGGAGAACGTGCCCGCGTTGCGCTGGAAGGAATTCGTGCGGCGCCTA
 350 GlyLeuSerAspHisGluIleAspArgLeuGluLeuGlnAsnGlyArgCysLeuArgGlu
 1135 GGGCTGAGCGACCACGAGATCGATCGGCTGGAGCTGCAGAACGGGCGCTGCCTGCCGCGAG
 370 AlaGlnTyrSerMetLeuAlaThrTrpArgArgArgThrProArgArgGluAlaThrLeu
 1195 GCGCAATACAGCATGCTGGCGACCTGGAGGCGGCGCACGCCCGCGCGGAGGCCACGCTG
 390 GluLeuLeuGlyArgValLeuArgAspMetAspLeuLeuGlyCysLeuGluAspIleGlu
 1255 GAGCTGCTGGGACCGCGTGTCTCCGCGACATGGACCTGCTGGGCTGCCTGGAGGACATCGAG
 410 GluAlaLeuCysGlyProAlaAlaLeuProProAlaProSerLeuLeuArg
 1315 GAGGCGCTTTGCGGCCCGCGCCCTCCCGCCCGCGCCAGTCTTCTCAGATGAGGCTGC
 1375 GCCCCTGCGGGCAGCTCTAAGGACCGTCTCGGAGATCGCCTTCCAACCCCACTTTTTTC
 1435 TGGAAAGGAGGGGTCTCTGCAGGGCAAGCAGGAGCTAGCAGCCCGCTACTTGGTGCTAAC
 1495 CCTCGATGTACATAGCTTTTCTCAGCTGCCTGCGCGCCGCGGACAGTCAGCGCTGTGCG
 1555 CGCGGAGAGAGGTGCGCCGTGGGCTCAAGAGCCTGAGTGGGTGGTTTGCGAGGATGAGGG
 1615 ACGCTATGCCTCATGCCCGTTTTGGGTGTCTCACCAGCAAGGCTGCTCGGGGGCCCTG
 1675 GTTCGTCCCTGAGCCTTTTTACAGTGCATAAGCAGTTTTTTTTGTTTTGTTTTGTTTT
 1735 GTTTTGTTTTTAAATCAATCATGTTACACTAATAGAACTTGGCACTCCTGTGCCCTCTG
 1795 CCTGGACAAGCACATAGCAAGCTGAAGTGTCTAAGGCAGGGGCGAGCACGGAACAATGG
 1855 GGCCTTCAGCTGGAGCTGTGGACTTTTGTACATACACTAAAATTCTGAAGTTAAAAAAA
 1915 AACCCGAATTC

Figur 2A

Fig.2a



Figur 2B

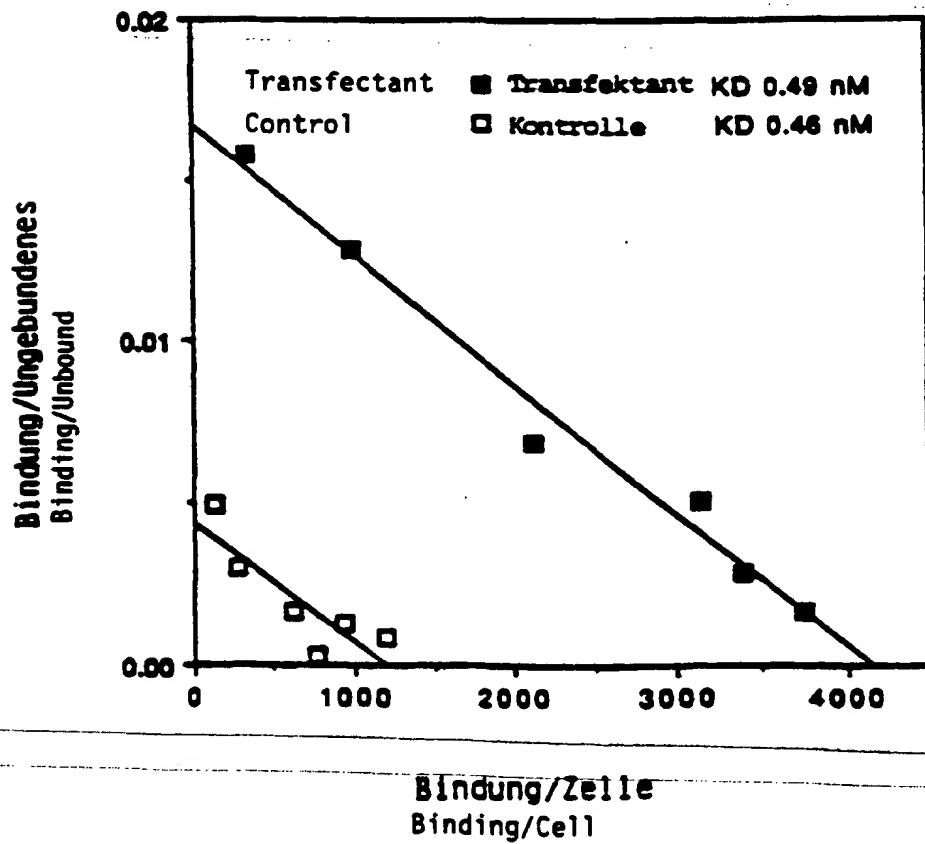


Figure 3 Fig.3

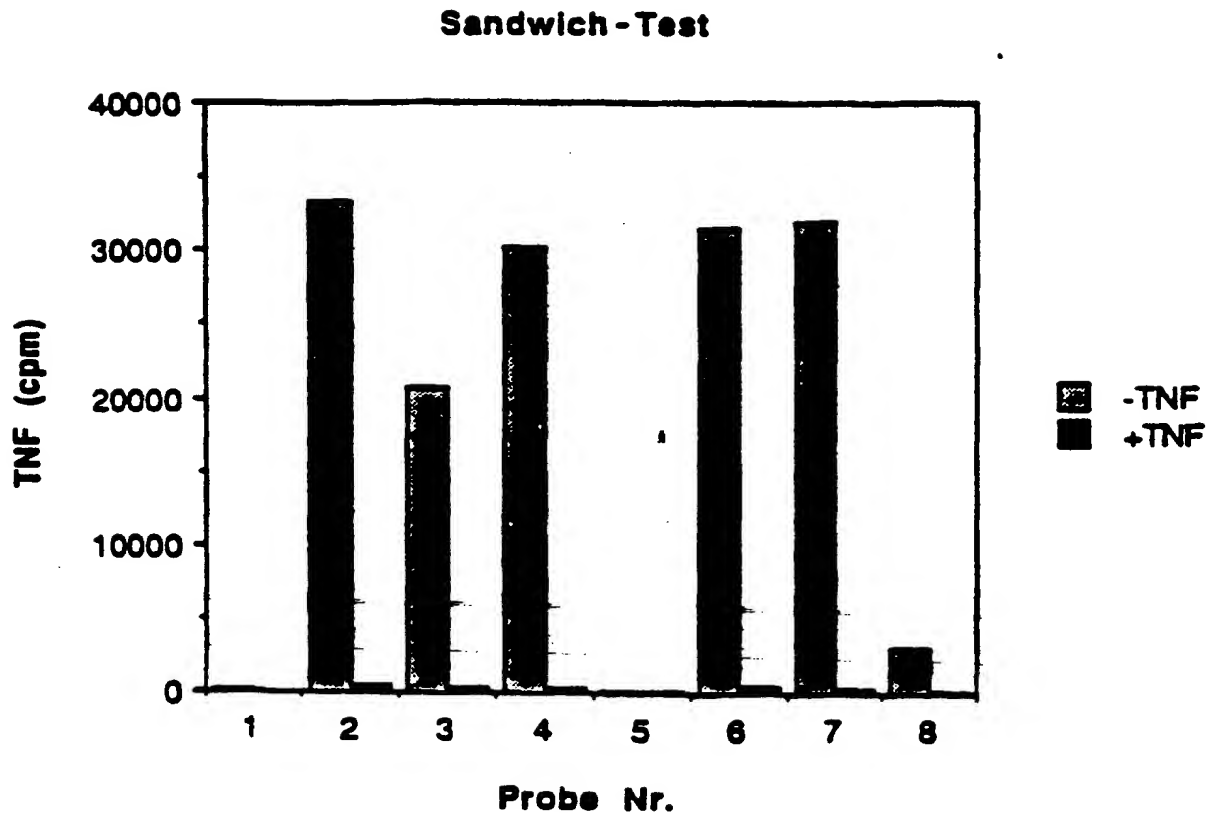


Figure 4 Fig.4

1 SerAspSerValCysAspSerCysGluAspSerThrTyrThrGlnLeuTrpAsnTrpVal
 1 TCGGACTCCGTGTGTGACTCCTGTGAGGACAGCACATACACCCAGCTCTGGAACTGGGTT
 21 ProGluCysLeuSerCysGlySerArgCysSerSerAspGlnValGluThrGlnAlaCys
 61 CCCGAGTGCTTGAGCTGTGGCTCCCGCTGTAGCTCTGACCAGGTGGAACTCARGCCTGC
 41 ThrArgGluGlnAsnArgIleCysThrCysArgProGlyTrpTyrCysAlaLeuSerLys
 121 ACTCGGGAACAGAACCGCATCTGCACCTGCAGGCCCGGCTGGTACTGCGCGCTGAGCAAG
 61 GlnGluGlyCysArgLeuCysAlaProLeuProLysCysArgProGlyPheGlyValAla
 181 CAGGAGGGGTGCCGGCTGTGCGCGCCGCTGCCGAAGTGCCGCCCGGGCTTCGGCGTGGCC
 81 ArgProGlyThrGluThrSerAspValValCysLysProCysAlaProGlyThrPheSer
 241 AGACCAGGAAGTGAACATCAGACGTGGTGTGCAGCCCTGTGCCCCGGGGACGTTCTCC
 101 AsnThrThrSerSerThrAspIleCysArgProHisGlnIleCysAsnValValAlaIle
 301 AACACGACTTCATCCACGGATATTTGCAGGCCCCACAGATCTGTAACGTGGTGGCCATC
 121 ProGlyAsnAlaSerArgAspAlaValCysThrSerThrSerProThrArgSerMetAla
 361 CCTGGGAATGCAAGCAGGGATGCAGTCTGCACGTCCACGTCCCCCAGCGAGTATGGCC
 141 ProGlyAlaValHisLeuProGlnProValSerThrArgSerGlnHisThrGlnProSer
 421 CCAGGGGCAGTACACTTACCCAGCCAGTGTCCACACGATCCCAACACACGCAGCCAGT
 161 ProGluProSerThrAlaProSerThrSerPheLeuLeuProMetGlyProSerProPro
 481 CCAGAACCCAGCACTGCTCCAGCACCTCCTTCTGCTCCCAATGGGCCCCAGCCCCCA
 181 AlaGluGlySerThrGlyAspPheAlaLeuProValGlyLeuIleValGlyValThrAla
 541 GCTGAAGGGAGCACTGGCGACTTCGCTCTTCCAGTTGGACTGATTGTGGGTGTGACAGCC
 201 LeuGlyLeuLeuIleIleGlyValValAsnCysValIleMetThrGlnValLysLysLys
 601 TTGGGTCTACTAATAATAGGAGTGGTGAAGTGTGTCATCATGACCCAGGTGAAAAGAG
 221 ProLeuCysLeuGlnArgGluAlaLysValProHisLeuProAlaAspLysAlaArgGly
 661 CCCTTGTCCTGCAGAGAGAGCCAGGTGCCTCACTTGCTGCCGATAGGCCCGGGGT
 241 ThrGlnGlyProGluGlnGlnHisLeuLeuIleThrAlaProSerSerSerSerSerSer
 721 ACACAGGGCCCCGAGCAGCAGCACCTGCTGATCACAGCGCCGAGCTCCAGCAGCAGCTCC
 261 LeuGluSerSerAlaSerAlaLeuAspArgArgAlaProThrArgAsnGlnProGlnAla
 781 CTGGAGAGCTCGCCAGTGCCTTGGACAGAGGGCGCCCACTCGGAACCAAGCCACAGGCA

APPENDIX C

Related Proceedings

None